Martek

Martek Biosciences Corporation

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February 9, 2000

Dockets Management Branch (HFA-305)
Food and Drug Administration
5630 Fishers Lane
Room 1061
Rockville, Maryland 20852

Re: Docket No. 96N-0391

To Whom It May Concern:

Please find enclosed, one copy of the document entitled "Opinion of an Expert Panel on the Generally Recognized as Safe (GRAS) Status of ARA and DHA Single Cell Oils for Infants and Children". This document is to be submitted to the Docket number mentioned above per request of Dr. David J. Kyle, Senior Vice President of Research & Development.

A copy of this document has been forwarded to Dr. Michael Falk, Director, Life Sciences Research Office (LSRO).

Please feel free to contact me should you have any questions.

Sincerely,

Joi Rivas

Joi Rivas
Administrative Assistant, Research & Development

Enclosure (1)

96N-0391

RPT 3

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OPINION OF AN EXPERT PANEL ON THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF ARA AND DHA SINGLE CELL OILS FOR INFANTS AND CHILDREN

GRAS Panel Evaluation of DHASCO® and ARASCO®
Martek Biosciences Corporation
December, 1999

OPINION OF AN EXPERT PANEL ON THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF ARA AND DHA SINGLE CELL OILS FOR INFANTS AND CHILDREN

EXECUTIVE SUMMARY

The undersigned, an independent panel of recognized experts (hereinafter the Panel), qualified by scientific and/or medical training and relevant international experience to evaluate the safety of food and food ingredients, was requested by Martek Biosciences Corporation to determine whether DHASCO® and ARASCO® oils (macronutrient oils produced by fermentation and containing docosahexaenoic acid (DHA) or arachidonic acid (ARA)), using scientific procedures, are Generally Recognized as Safe (GRAS) for consumption by infants (birth to 1 year) and children (1-12 years) at dose levels up to 2.5% each of total dietary fat. For infants, this dose is equivalent to 1.25% of dietary calories or about 150 mg of the oil/kg body wt/day. A comprehensive search of the scientific literature (published and unpublished) for information on the use and safety of ARASCO and DHASCO through October 15, 1999 was conducted by Martek and was made available to the Panel. The Panel members independently and critically evaluated the materials submitted by Martek Biosciences Corporation and other materials deemed appropriate and conferred by telephone several times before preparation of this report.

Earlier safety concerns regarding growth of infants fed fish oil-supplemented formulas have been addressed. They appear to be associated with the eicosapentaenoic acid (EPA) present in the fish oils. EPA leads to a depression of ARA and associated eicosanoid levels. In contrast, growth inhibition has never been observed when DHASCO, which does not contain EPA, was used as a dietary DHA source for infants. The growth delay seen in some fish oil-based formula studies can be avoided by adding an ARA source along with the DHA to infant formulas. Recent controlled studies indicate that preterm and term infants fed formulas fortified with both ARASCO and DHASCO oils have better growth indices than infants fed unsupplemented formula. Other potential safety concerns associated with fish oil, such as increased bleeding times, have never been linked to the use of DHASCO or ARASCO oils.

The Panel first considered the importance of DHA and ARA for infants and children. After reviewing the literature regarding the efficacy of DHA and ARA (from any source) in infant nutrition, the Panel unanimously agrees that: 1) there is a deficiency in the DHA and ARA status in infants fed formula not fortified with DHA and ARA; 2) that this is reflected in a decreased blood and other tissue (e.g., brain) levels of DHA and ARA; and 3) that this deficiency contributes to the visual and neurological deficits observed in formula-fed compared with breast-fed infants. The link between these two long-chain polyunsaturated fatty acids and developmental outcome has been established by well-controlled clinical studies indicating that addition of DHA and ARA to the formula not only corrects the deficiency, but can also contribute to the elimination of these neurological deficits.

This Panel also considered the opinion of other Expert Panels, associated with prestigious scientific and medical organizations, that convened over the last ten years to consider the question of whether to add DHA and ARA to infant formulas. The general consensus among the different panels was that both of these fatty acids should be added to formulas at levels corresponding to those found in human breast milk. One panel from the Life Science Research Organization (LSRO) of the American Society for Nutritional Sciences, that reviewed the addition of long-chain fatty acids as part of an overall review

of the requirements for term (not preterm) infants formulas did not recommend that DHA and ARA be a required addition to term formulas at this time. However, they did recognize the importance of maintaining the DHA status of infants and suggested that supraphysiological levels of linolenic acid (a precursor to DHA) should be added to help improve the DHA status of the formula-fed infant. This LSRO Panel did not review several of the most recent studies, and recommended a re-evaluation of this issue in the near future as new clinical study data emerge. Another LSRO panel is presently considering the DHA and ARA requirements for preterm infants.

The chemical composition and manufacturing processes for both DHASCO and ARASCO oils were carefully reviewed by the Panel to ensure that oil quality meets standards for food production. DHASCO oil is a triglyceride, produced by the alga *Crypthecodinium cohnii*, which contains 40% by weight of DHA (specifications given in section 6). ARASCO oil, also predominantly triglyceride, is produced by the fungus *Mortierella alpina* and contains 40% by weight of ARA. In both cases, these oils contain DHA and ARA in triglyceride structures that are chemically equivalent to those delivered to infants from mother's milk. Both oils also contain other common saturated and monounsaturated fatty acids. Minor nonsaponifiable fractions of the oils have been characterized and contain primarily cholesterol-related sterols commonly found in other food sources. Both oils are manufactured by a controlled fermentation process, followed by oil extraction and purification using methods common to the vegetable oil industry. All ingredients used in the processing of the oils are either food grade, or of higher quality, and the entire process meets current Good Manufacturing Practices for foods. All oils undergo rigorous analytical and quality assurance testing and meet well-defined product specifications prior to release.

Based on elevation of blood lipid levels following ingestion of either DHASCO or ARASCO, both oils are absorbed in a manner consistent with other dietary triglycerides. The DHA and ARA are distributed throughout the body and are found at the highest levels in brain, retina, testes, and heart. DHA and ARA can be catabolized completely to CO2 and H2O, but the catabolic rate is slower than with other dietary fatty acids. This is necessary in order to maintain DHA and ARA levels in the rapidly expanding neurological tissues of infants and children. In addition, ARA, but not DHA, serves as a precursor molecule to eicosanoids, and the omega-6 series of eicosanoids are well recognized as stimulators of immune function. Studies have shown that small amounts of DHA can be retroconverted to EPA in humans, although accumulation of EPA is negligible at doses of DHASCO used for infant supplementation. Although oxidation of these highly unsaturated fatty acids in blood or tissues has been raised as a potential concern, studies have shown that DHA, in particular, activates antioxidant systems in the body and may actually protect against oxidation of polyunsaturated fatty acids. Animal and human studies have confirmed that supplementation with DHA and ARA protects, rather than accentuates oxidative damage. This is not inconsistent with the finding that infants who receive breast milk (containing DHA and ARA) are more protected from Necrotizing enterocolitis (NEC) than infants fed formulas without DHA and ARA.

A large number of safety studies have been conducted using DHASCO and ARASCO oils, including acute, subchronic, developmental and reproductive toxicology studies in rats and *in vitro* mutagenicity assays. All studies were modeled after FDA

Redbook guidelines and conducted at GLP-compliant laboratories. The study results were evaluated relative to well known effects of supplementation with high doses of polyunsaturated fatty acids (PUFAs) in order to distinguish between PUFA-related effects and effects due to the sources themselves. None of the studies indicated that the oils were toxic, and the No Adverse Effect Levels (NOAELs) were determined to correspond to the highest doses tested. Modestly increased liver weights were noted, but this was not a consistant finding, nor was it accompanied by abnormal histology or serum enzyme levels. Furthermore, the liver weight changes were no longer apparent when assessed relative to other organ weights (*e.g.* brain). These changes were, therefore, deemed not to be toxicological in nature as this is a well-known effect of the Long Chain PUFAs in mammals when administered at high dose levels, well above those intended for infants or children. All other concerns raised by previous reviews have been addressed in this document (Appendix 4). In addition, the crude biomasses containing the DHASCO or ARASCO have also been extensively tested and found to be nontoxigenic.

Studies conducted in twelve different animal species, including nonhuman primates, have provided a large base of experience and extensive toxicological data with DHASCO and ARASCO oils (summarized in Appendix 1). None of the reports (published or unpublished) have suggested any toxicological or safety issues associated with the use of these oils. At least fourteen well-controlled clinical studies involving over 1500 infants have confirmed that DHASCO and ARASCO increase circulating levels of DHA and ARA in preterm and term infants (summarized in Appendix 2). Significant improvements in growth, visual and mental acuity have also been reported in infant groups supplemented with these oils and no adverse events have been reported. In addition, twenty-nine separate, well-controlled clinical intervention studies using DHASCO and/or ARASCO have been conducted on adults or children with no reported adverse effects of the treatment (summarized in Appendix 3). Two such studies with particular emphasis on safety and bioavailability were conducted by the U.S. Department of Agriculture using high doses of either ARASCO (3 g/day) or DHASCO (15 g/day) with adult volunteers. The preclinical and clinical studies conducted with these oils further support their use as a safe dietary source of DHA and ARA.

In addition, these oils have now been in commercial use in infant formulas in over 60 countries around the world (including the United Kingdom, France and Israel) at levels in accord with WHO/FAO guidelines for as long as three years with no reported adverse findings during that time. Over 40 million capsules have been sold as dietary supplements to an estimated 250,000 individuals, primarily in the United States, with no significant adverse events reported to the Company. The large numbers of individuals (infants through adults) who have consumed the DHASCO or ARASCO oils as commercial products or in clinical trials with no adverse effects provides additional support for the establishment of GRAS status for these products at use levels that can commonly be obtained in the diet.

Human breast milk is the "Gold Standard" for infant nutrition. In addition to other important nutrients, human breast-fed infants receive DHA- and ARA-containing triglycerides from their mother's milk. Based on this fact, the growing body of evidence demonstrating the importance of supplying infants with DHA and ARA, and after a critical evaluation and analysis of the safety and clinical information available on DHASCO and ARASCO as DHA- and ARA-containing triglycerides, the undersigned Expert Panel has determined that these oils, derived from the referenced algal and fungal sources, meeting food grade specifications and produced according to current Good Manufacturing Practices (cGMP; 21 CFR 182.1) to be Generally Recognized as Safe (GRAS) for use in supplementing the diets of infants and children up to 15 years of age at levels of 2.5% of dietary fat (1.25% of energy or up to 150 mg DHASCO (or ARASCO) per kg body weight per day).

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1. INTRODUCTION

An independent panel of recognized experts (hereinafter the Panel), qualified by scientific and/or medical training and relevant international experience to evaluate the safety of food and food ingredients, was requested to determine whether DHASCO® and ARASCO® oils (macronutrient oils produced by microbial fermentation and containing docosahexaenoic acid (DHA) or arachidonic acid (ARA)), using scientific procedures, are Generally Recognized as Safe (GRAS) for consumption by infants (birth to 1 year) and children (1-12 years) at dose levels up to 2.5% of total dietary fat. In an infant, this dose is equivalent to 1.25% of dietary calories or about 150 mg of the oil/kg body wt/day. A comprehensive search of the scientific literature (published and unpublished) for information on the use and safety of ARASCO and DHASCO through October 15, 1999 was conducted by Martek Biosciences Corporation (Martek) and was made available to the Panel. Reprints, abstracts, laboratory data and other materials were also provided. The Panel members independently and critically evaluated the materials submitted by Martek and other materials deemed appropriate. The Panel conferred by telephone several times and also conferred with members of Martek Biosciences Corporation for issues requiring clarification before the preparation of this report, which includes a review of the pertinent literature and recommendations.

2. THE IMPORTANCE OF DHA AND ARA IN INFANT NUTRITION

Prior to assessing the safety of the reference materials DHASCO and ARASCO oils, the Panel considered the importance of dietary DHA and ARA from any source for infants and children. The Panel unanimously agrees that there is a biochemical deficiency in infants receiving formulas with no supplemental DHA and ARA relative to infants being fed their own mother's milk -- the gold standard for infant nutrition. This biochemical deficiency is reflected in abnormally low levels of circulating DHA and ARA in the blood of unsupplemented formula-fed babies relative to breast-fed babies, and there is clinical evidence to suggest that there may be both short term and long term detrimental consequences as a result of this deficiency.

2.1 DHASCO AND ARASCO ARE SUBSTANTIALLY EQUIVALENT TO DHA AND ARA TRIGLYCERIDES IN HUMAN MILK.

From an evolutionary point of view, breast milk represents the optimal source of nutrition for the human infant and it is often referred to as the "gold standard". Infant formulas are also used as the sole source of nutrition for a human infant and should, therefore, be as nutritionally balanced as human milk. DHA and ARA are found in human milk in low, but significant quantities. The DHA and ARA content of human milks from 65 published reports around the world are given in Table 2.1-1. It is clear that the DHA content of human milk is quite variable ranging from 0.06% to 1.4% of total fat and has been shown to be dependent on the dietary DHA intake of the mother (165). Mothers with diets low in fish and other sources of DHA, but otherwise high in fat (e.g., a typical Western diet), have breast milk DHA levels on the low end of the range. Women from the United States, for example, have

among the lowest levels of DHA in their breast milk compared to worldwide averages (Figure 2.1-1).

To determine the optimal level of DHA in breast milk we must consider the diets to which our species evolved. Such Paleolithic diets were thought to contain much more DHA and much less total fat than the typical Western diet (32), and the DHA content of breast milk would therefore have been much higher than it is today. Thus, an estimated optimal level of DHA in the breast milk would likewise be much higher than it is in the United States today.

Table 2.1-1 Breast milk DHA and ARA levels from women around the world.

Author, year	*Reference*	*wt % DHA*	*wt % ARA*	*Country*
Finley, et al. (1985)	(89)	0.06	0.29	USA
Harris, et al. (1984)	(114)	0.1	0.4	USA
Putnam, et al. (1982)	(198)	0.1	0.6	USA
van der Westhuizen, et al. (1988)	(235)	0.1	1	S. Africa
Sas, et al. (1986)	(208)	0.1	0.5	Hungary
Spear, et al. (1992)	(221)	0.11	0.54	USA
Sanders and Reddy (1992)	(207)	0.14	0.32	UK (vegan)
Maurage, et al. (1998)	(171)	0.14	0.24	France
Auestad, et al. (1997)	(9)	0.15	0.48	USA
Spear, et al. (1992)	(221)	0.15	0.58	USA
Okolska, et al. (1983)	(189)	0.15	1.56	Poland
Dotson, et al. (1992)	(80)	0.16	0.53	USA
Jackson, et al. (1994)	(134)	0.16	0.56	USA
Harzer, et al. (1983)	(115)	0.16	0.39	Germany & UK
Carlson, et al. (1986)	(44)	0.19	0.59	USA
Francois, et al. (1998)	(92)	0.2	0.5	USA
van der Westhuizen, et al. (1988)	(235)	0.2	0.6	S. Africa
Innis, et al. (1994)	(132)	0.2	0.5	Canada
Bitman, et al. (1983)	(23)	0.21	0.58	USA
Henderson, et al. (1998)	(118)	0.21	0.52	USA
Drury and Crawford (1990)	(81)	0.21	0.6	Hungary
Makrides, et al. (1995)	(163)	0.21	0.4	Australia
Makrides, et al. (1996)	(165)	0.21	0.41	Australia
Koletzko, et al. (1988)	(147)	0.22	0.36	Germany
Bitman, et al. (1983)	(23)	0.23	0.6	USA
Sanders, et al. (1978)	(206)	0.23	0.72	UK (vegan)
Genzel-Boroviczeny, et al. (1997)	(94)	0.23	0.45	Germany
Bitman, et al. (1983)	(23)	0.24	0.55	USA
Beijers and Schaafsma (1996)	(16)	0.24	0.31	Netherlands
Genzel-Boroviczeny, et al. (1997)	(94)	0.24	0.48	Germany
Martin, et al. (1993)	(170)	0.24	0.36	France
Carnielli, et al. (1998)	(51)	0.26	0.48	Netherlands
Foreman-van Drongelen, et al. (1996)	(91)	0.26	0.52	Netherlands
van Beusekom, et al. (1993)	(233)	0.26	0.47	Netherlands
Muskiet, et al. (1987)	(181)	0.27	0.6	Tanzania
Specker, et al. (1987)	(222)	0.29	0.69	USA
Hall (1979)	(111)	0.29	0.19	UK
Yu, et al. (1998)	(257)	0.29	0.46	Sweden

Author year	*Reference*	*wt % DHA*	*wt % ARA*	*Country*
Sanders and Reddy (1992)	(207)	0.3	0.38	UK
Jansson, et al. (1981)	(137)	0.3	0.4	Sweden
Villacampa, et al. (1982)	(240)	0.3	0.57	Spain
Cherian and Sim (1996)	(56)	0.3	0.4	Canada
Clandinin, et al. (1997)	(61)	0.3	0.54	Canada
Babin et al. (1999)	(10)	0.31	0.5	France
Kaila, et al. (1999)	(142)	0.31	0.35	Finland
Rueda, et al. (1998)	(202)	0.32	0.52	Panama
Billeaud, et al. (1997)	(19)	0.32	0.52	France
Chardigny, et al. (1995)	(55)	0.32	0.5	France
Gibson, et al. (1981)	(95)	0.32	0.4	Australia
FAO/WHO RECOMMENDATIONS FOR FULL TERM INFANTS	(84)	**0.33**[1]	**0.65**	
de la Presa-Owens, et al. (1998)	(71)	0.34	0.5	Spain
Ogunleye, et al. (1991)	(188)	0.34	0.56	Nigeria
Beijers and Schaafsma (1996)	(16)	0.34	0.37	Netherlands
ISSFAL 1999 RECOMMENDATIONS FOR FULL TERM INFANTS	(133)	**0.35**	**0.50**	
Sanders and Reddy (1992)	(207)	0.37	0.35	UK
Guesnet, et al. (1993)	(110)	0.37	0.45	France
Rueda, et al. (1998)	(202)	0.38	0.69	Spain
Prentice, et al. (1989)	(197)	0.39	0.31	The Gambia
Luukkainen, et al. (1994)	(162)	0.39	0.37	Finland
de Lucchi, et al. (1988)	(72)	0.4	0.8	Spain
Jacobs, et al. (1996)	(135)	0.4	0.6	Netherlands
van Beusekom, et al. (1993)	(233)	0.4	0.5	Dominica
Clandinin, et al. (1981)	(58)	0.4	0.5	Canada
Innis, et al. (1988)	(126)	0.4	0.7	Canada
Muskiet, et al. (1987)	(181)	0.41	0.56	Surinam
Horby Jorgensen, et al. (1996)	(124)	0.43	0.47	Sweden
Muskiet, et al. (1987)	(181)	0.43	0.71	Curacao
Luukkainen, et al. (1995)	(162)	0.48	0.54	Finland
Drury and Crawford (1990)	(81)	0.49	0.57	Thailand
Innis, et al. (1990)	(130)	0.5	0.8	Canada
Ogunleye, et al. (1991)	(188)	0.53	0.36	Japan
Fidler, et al. (1998)	(87)	0.55	0.77	Germany
Rocquelin, et al. (1998)	(200)	0.55	0.44	Congo
Boersma, et al. (1991)	(25)	0.56	0.58	St. Lucia
Sanders, et al. (1978)	(206)	0.59	0.54	UK
Kneebone, et al. (1985)	(144)	0.71	0.64	Malaysia
Kneebone, et al. (1985)	(25)	0.9	0.47	Malaysia
Kneebone, et al. (1985)	(25)	0.9	0.57	Malaysia
Koletzko, et al. (1991)	(149)	0.93	0.82	Nigeria
Innis, et al. (1988)	(126)	1.4	0.6	Canada

[1] Fatty acid recommendations given in mg/kg body weight and were converted to weight % fat assuming that term infants consume 110 kcal/kg body wt/day and that 50% of calories in formula are from fat.

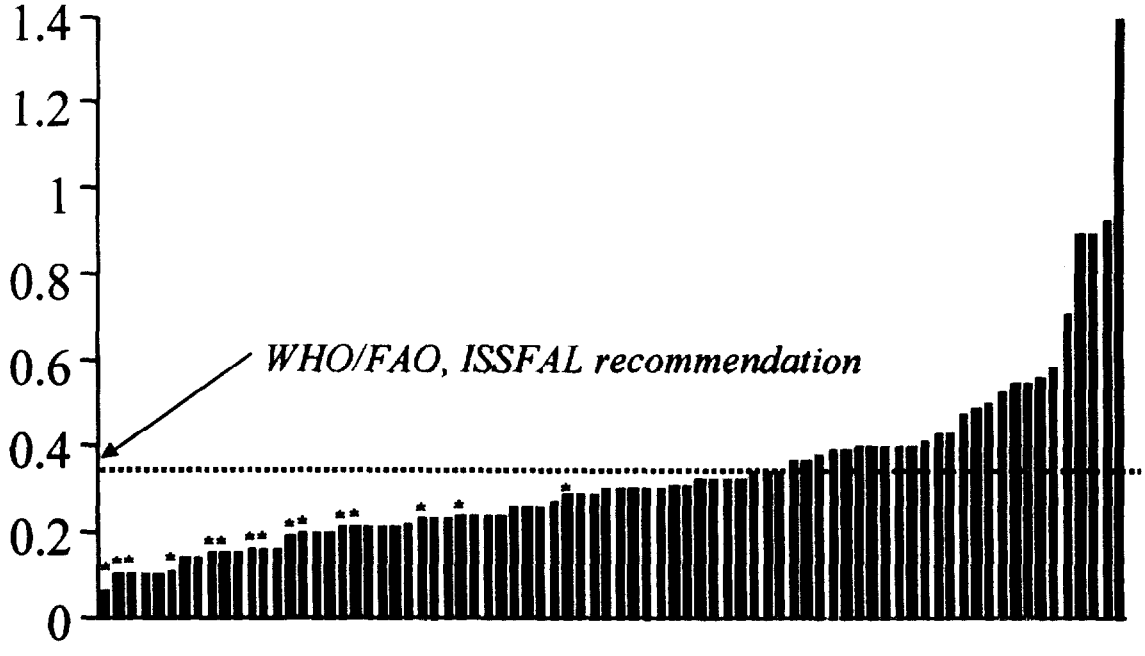

Figure 2.1-1. DHA content (% of total milk fat) from the 65 separate references in Table 2.1-1 arranged in ascending order. US values are given in red (stars).

DHA and ARA are found in both triglycerides and phospholipids in human milk. However, breast milk fat is primarily triglyceride (*ca.* 98%), with only about 1% phospholipid, and 1% nonsaponifiable fats such as cholesterol and phytosterols (141). The DHA level in the phospholipid fraction of breast milk is generally higher than in the triglyceride fraction (1.4% vs. 0.2% respectively) (141) but, because there is much more triglyceride, the vast majority of the DHA being delivered to the infant from breast milk is in the triglyceride form, rather than the phospholipid form. Even though the bulk triglyceride may be only 0.2% DHA, the lowest DHA content of a triglyceride molecule would be 33% (*i.e.*, one DHA per three fatty acids on the triglyceride). This DHA molecule may be on the outside position of the triglyceride (sn-1 or sn-3) or on the inside position (sn-2) (see Figure 2.1-2). If it is on the sn-1 or sn-3 position, it will be cleaved by the infant's pancreatic lipases and enter the gut wall as a free fatty acid. If it is on the sn-2 position, it is efficiently absorbed as the sn-2 monoglyceride.

The DHASCO oil contains about 40-50% DHA by weight. Therefore, the most abundant triglyceride structure also has only a single DHA per triglyceride. However, there is also a possibility of two DHA molecules on some triglycerides in DHASCO (Figure 2.1-2). Even if that is the case, however, the DHA will still be absorbed either as the free fatty acid or as the monoglyceride (in a fashion identical to that of the DHA-triglyceride from mother's milk) after processing by the baby's lipase in the gut. This same discussion must also be considered when comparing equivalence of the ARA in ARASCO to the ARA in the ARA-triglycerides from mother's milk (Figure 2.1-2).

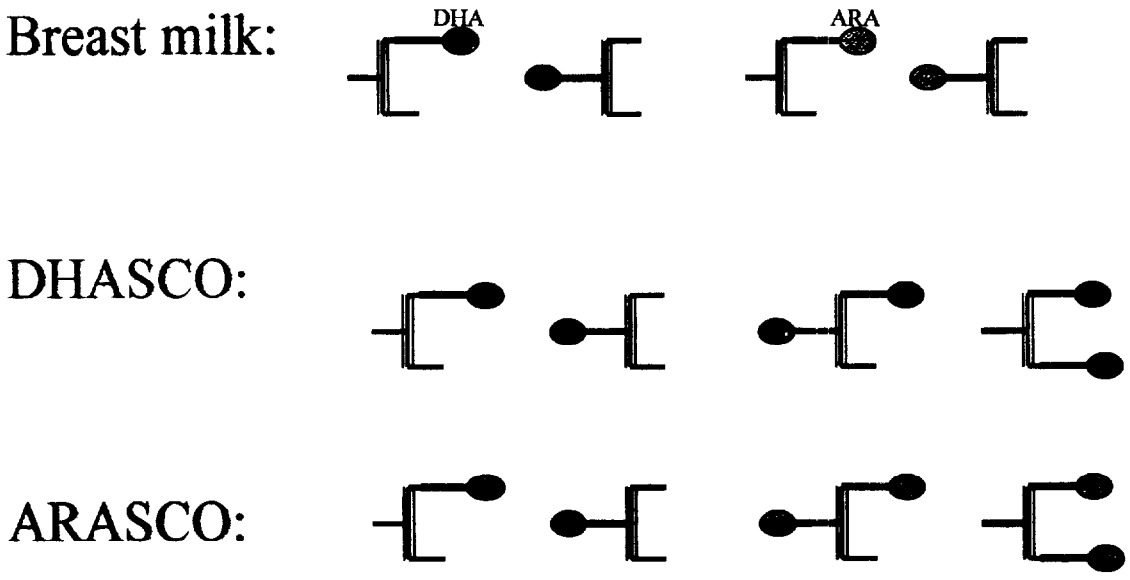

Figure 2.1-2. Triglyceride structures in human breast milk and DHASCO and ARASCO prior to cleavage in the gut of an infant by lipases.

Over the past twenty years, there have been a large number of retrospective studies comparing the neurological outcomes of breast-fed and formula-fed infants. A recent meta-analysis of the most relevant of these studies has indicated that there is a consistent 3-4 IQ point advantage to the breast-fed infants even after the contributions of all other recognized confounding factors had been removed (5). A summary of the data from this paper indicates a consistency across the studies in the meta-analysis (Figure 2.1-3a) as well as a dose response in terms of duration of breast feeding (Figure 2.1-3b). This latter observation specifically implicates some factor in the source of nutrition as being a causative agent for the improved intelligence scores later in life. Several researchers have speculated that LC-PUFAs, or specifically DHA, may be that factor (6, 125). Breast-fed babies, however, are getting many nutrients from the breast milk besides DHA and ARA, and one can argue that, based on these data alone, the specific contribution of DHA and ARA to improved long term IQ is inconclusive. One observation, however, is very clear and consistent: infants who are provided standard infant formulas (*i.e.*, without supplemental DHA and ARA) have significant deviations in their blood and brain biochemistry relative to breast-fed babies.

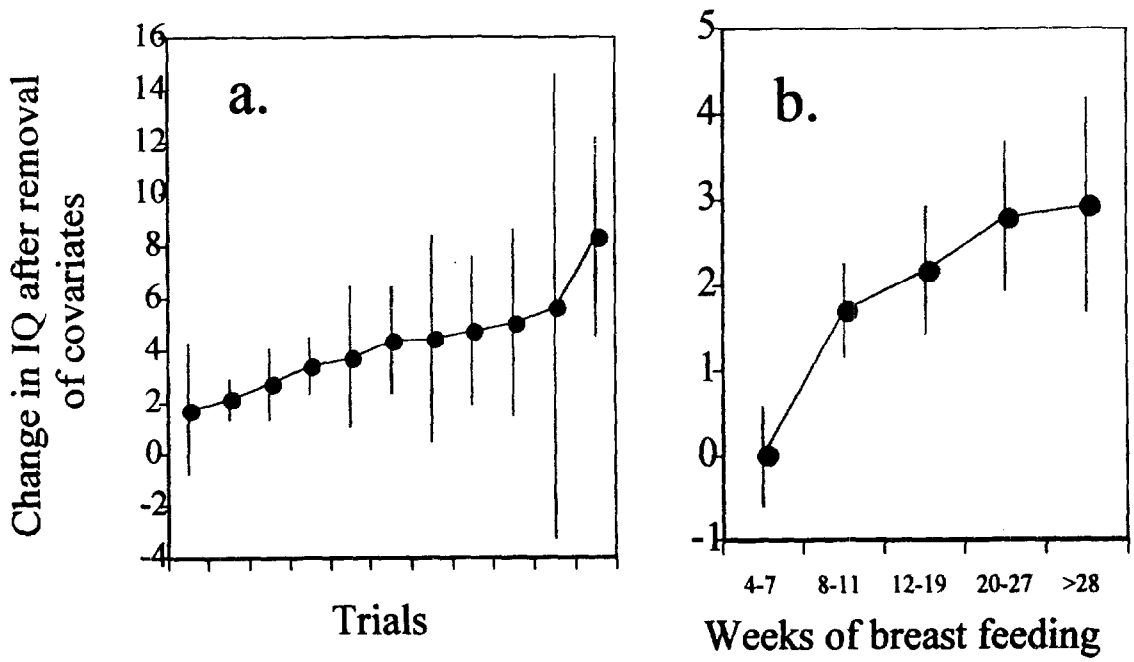

Figure 2.1-3. Meta-analysis of a group of studies comparing the neurological outcome of breast-fed vs. formula-fed infants. (a) differences in IQ of children who were breast-fed as babies vs. a formula-fed group after the contributions of various influential covariates have been removed; and (b) the effect of duration of breast-feeding on the IQ difference between breast-fed and formula-fed infants. Data is from Anderson et al (6)

2.2 BIOCHEMICAL OUTCOMES. Preterm and full term infants fed standard, unsupplemented formulas have a circulating DHA and ARA status (as indicated by red blood cell or plasma phospholipid levels of these fatty acids) about one-half that of breast-fed infants (3, 22, 24, 40, 50, 60, 61, 91, 96, 109, 121, 123, 128, 145, 148, 163, 171). Furthermore, the brain DHA levels of formula-fed infants are about one-third lower than those of breast-fed infants (85, 164). Table 2.2-1 lists 32 controlled clinical studies involving over 2,600 infants (over 1,300 received the LC-PUFA-supplemented formulas) performed over the last 15 years (17 studies with term infants and 15 studies with pre-term infants). Each study compared outcomes of standard formula-fed infants with DHA-supplemented formula-fed infants and, in most instances, also with breast-fed infants. In every study reporting blood fatty acids levels, the DHA status of the infants was returned to normal (as defined by the DHA status of breast-fed infants) when the formulas were supplemented with DHA. In all of these studies, the ARA levels were also normalized when supplemental ARA was used in the formulas as well. Of all the trials completed with DHA/ARA supplementation in Table 2.1-1, more infants have received DHASCO or ARASCO (573 infants) than other sources of DHA and ARA (378 received egg yolk and 417 received various fish oils).

Since it can be argued that these changes in the blood and brain biochemistry in the formula-fed infant may not be important, it is crucial to more fully understand the function of DHA in the tissues of the body. Recent studies have revealed that DHA has many critical functions in the normal development and metabolism of neuronal cells. These include, but are not limited to: 1) the control of normal migration of neurons from the surface of the ventricles of the brain to the cortical plate during brain development (253); 2) the control of the normal resting potential of the neurons and cardiac cells by regulation of sodium and calcium channels (252); 3) the regulation of the packing density of certain membrane proteins such as rhodopsin in the retina (229); and, 4) the regulation of levels of certain adrenergeneric and serotonergic neurotransmitters (74, 119). With such key roles in normal neuronal development and function, it is quite plausible that abnormally low levels of this primary nutrient, DHA, during the development of the brain, could lead to the long term neurological deficits observed in formula-fed infants relative to breast-fed infants (125, 160).

In many of the clinical trials listed in Table 2.2-1, the study protocols involved using the supplemented formulas only for the first 30-120 days before switching back to routine formulas. Interestingly, blood lipid assessments at one year of age have shown that the babies who had the DHA/ARA supplemented formulas maintained their high DHA status in spite of many months on unsupplemented formulas in a fashion similar to the breast fed babies. It has also recently been shown that children with attention deficit hyperactivity disorder (ADHD) also have a low DHA status relative to a pair-matched normal cohort, even though there were no differences in diet (227). The authors also demonstrated a significant correlation between formula feeding and the low DHA status at 8-12 years of age (and formula feeding and ADHD). These data collectively suggest that a "nutritional programming" event is taking place during the first few months of life, wherein the absence of DHA during this crucial period, programs a low DHA status to the individual for many years to follow.

2.3 VISUAL OUTCOMES. Of the 32 DHA/ARA supplementation studies shown in Table 2.1-1, thirteen analyzed visual outcomes (*e.g.*, visual acuity as measured by visual evoked potential or Teller Acuity Cards) as a primary endpoint. Nine of these studies included a breast-fed infant control group (9, 40, 41, 67, 68, 96, 112, 121, 123, 163, 166). In every study that reported a difference in visual acuity between breast-fed and formula-fed infants (22, 40, 121, 163) (*i.e.*, where the babies fed standard formula had a visual deficit compared to breast-fed babies -- the gold standard), this deficit was overcome by adding DHA/ARA to the infant formula. That is, the infant groups receiving DHA/ARA-supplemented formulas where DHA and ARA were provided at the same levels as found in breast milk did not display this deficit, and they had significantly improved visual responses compared to babies from the unsupplemented formula-fed group. The magnitude of the visual improvement in one study of term infants supplemented with DHASCO and ARASCO was equivalent to "one line on an eye chart" after one-year (22).

Table 2.2-1 Thirty-two infant clinical intervention trials[2] with DHA/ARA-supplemented formulas provided by egg yolk, fish oil, or DHASCO/ARASCO single cell oils (SCO).

Study	Ref.	PUFA Source	Study Size (n)	Suppl'd Infants (n)	Are Babies' Blood Lipids Normalized with DHA/ARA?		Are Functional Outcomes Normalized with DHA/ARA?	
					DHA	ARA	Visual	Neurological
Preterm Studies								
Koletzko (1989)	(148)	egg	29	8	yes	yes	not tested	not tested
Clandinin (1992)	(59)	fish	32	12	yes	no	not tested	not tested
Hoffman (1993)	(121)	fish	51	28	yes	no	**yes**	not tested
Carlson (1993)	(48, 246)	fish	67	33	yes	no	**yes**	**yes**
Carnielli (1994)	(50)	SCO	16	5	yes	yes	not tested	not tested
Foreman (1996)	(91)	SCO	43	15	yes	yes	not tested	not tested
Boehm (1996)	(24)	egg	41	12	yes	yes	not tested	not tested
Carlson (1996)	(46, 49)	fish	59	26	yes	no	**yes**	**yes**
Koletzko (1996)	(67)	egg	57	18	n.g.	n.g.	no difference	**yes**
Hansen (1997)	(112)	SCO	284	113	yes	yes	no difference	not tested
Clandinin (1997)	(61)	SCO	91	48	yes	yes	not tested	not tested
Vanderhoof (1997)	(237)	SCO	287	60	yes	yes	not tested	not tested
Carnielli (1998)	(51)	SCO/egg	77	38	n.g.	n.g.	not tested	not tested
Carlson (1998)	(43)	egg	119	34	yes	yes	not tested	not tested
Ryan (1999)	(203)	fish	63	31	yes	no	not tested	not tested
Term Studies								
Kohn (1994)	(145)	egg	n.g.	n.g.	yes	yes	not tested	not tested
Agostoni (1995)	(2, 3)	egg	86	27	yes	yes	not tested	**yes**
Decsi (1995)	(73)	egg	22	12	yes	yes	not tested	not tested
Makrides (1995)	(163, 166)	fish	55	12	yes	no	**yes**	not tested
Carlson (1996)	(40)	egg	58	19	yes	yes	**yes**	not tested
Innis (1996)	(128)	fish	131	68	yes	no	not tested	not tested
Koletzko (1996)	(68)	egg	53	32	n.g.	n.g.	no difference	**yes**
Gibson (1997)	(96)	SCO	113	45	yes	yes	not tested	not tested
Auestad (1997)	(9, 212)	egg	197	89	yes	yes	no difference	no difference
Gibson (1997)	(96)	fish	67	40	n.g.	n.g.	no difference	not tested
Bellu (1997)	(17)	egg	123	60	n.g.	n.g.	not tested	not tested
Willatts (1998)	(248)	egg	44	21	n.g.	n.g.	not tested	**yes**
Birch (1998)	(22)	SCO	108	45	yes	yes	**yes**	**yes**[4]
Horby (1998)	(123)	fish	56	26	yes	no	no difference	not tested
Maurage (1998)	(171)	fish	83	47	yes	yes	not tested	not tested
Carlson (1999)	(41)	fish/SCO	335	223	yes	yes	no difference	no difference
Makrides (1999)	(167)	Fish/egg	146	55	Yes	Yes	not tested	not tested

SCO, single cell oil (in all cases DHASCO and ARASCO were added together); n.g.,not given.

[2]Trials that have been published as full manuscripts or abstracts.

[3] Total number of infants enrolled in the study.

[4] The results of the cognitive portion of the study are unpublished, but were presented at the a ISSFAL/NIH workshop in Washington D.C. in April, 1999 and are in press (ref. 21).

Two additional studies (in which there was no breast milk control) showed improved visual acuity in infants fed DHA-supplemented formulas compared to infants fed standard formula. In most of the studies the formula-fed infants eventually "caught up" with the breast-fed infants when the cruder measures of visual acuity were used. However, differences were still evident even after three years of age when finer measures of visual perception, such as stereo acuity were used (20). Among the remaining studies, no statistically significant differences were found between formula-fed and breast-fed babies using the test metrics employed and, therefore, no effect of DHA/ARA supplementation was observed. A closer look at the experimental design in the studies not showing an improvement in the DHA/ARA supplementation group may at least in part explain the lack of statistically significant effects of DHA/ARA supplementation. For example, in one of the cases (9), only a small amount of DHA was used in the supplementation (less than one third the level of the typical recommendations outlined in section 2.8 below), and the breast milk DHA content of the nursing mothers was remarkably low compared to worldwide norms (see Table 2.1-1 and Figure 2.1-1). In another case (112), the period of supplementation was short (average one month - during hospital stay of the preterm infant). In the other cases, the sample size may simply have been too small, the variability too large, or the test metric not sensitive enough to identify any significant differences between groups. This is particularly obvious in Horby-Jorgensen *et al.* (123) where there was a clear trend to improved visual acuity, but the difference did not reach statistical significance with the small sample size.

2.4 NEUROLOGICAL OUTCOMES. Of the 32 DHA/ARA supplementation studies shown in Table 2.2-1, nine reported neurological outcomes (e.g., tests for visual memory and attention, problem solving or standardized developmental tests like the Bayley Scales of Infant Development) as a primary endpoint. Six of those nine reported statistically significant improvements in neurological/cognitive outcomes in infants fed formulas supplemented with DHA or DHA/ARA compared to infants fed standard formula. As with visual outcomes, in every study detecting a deficit in standard formula-fed infants compared to breast-fed infants, the deficit was overcome by including DHA/ARA in the infant formula, and the babies receiving the DHA/ARA-supplemented formulas performed significantly better than the infants receiving standard formula. One of the studies (9, 212) which did not show a statistically significant difference in neurological outcome between formula-fed (supplemented or unsupplemented) and breast-fed babies, as mentioned above, had only a very low level of DHA available to the babies either in the supplemented formulas or in the breast milk of the nursing mothers, and consequently, no difference was detected between any of the groups. One other study (41) showed a clear trend towards improved Bayley scores in the DHA/ARA supplemented infants, but the difference did not reach statistical significance at $p<0.05$. New data on neurological assessments in the study by Birch and colleagues (22) was recently presented at a workshop in Washington D.C. (217) and is included in Table 2.2-1. These data (21) indicated that at the 18-month assessment of these babies, the infants fed standard formula exhibited a statistically significant seven-point deficit in developmental

quotient on the Bayley Mental Development Index assessment relative to infants fed DHASCO/ARASCO-supplemented formulas. Once again, the standard formulas were shown to result in a neurological deficit that was overcome by the addition of DHASCO and ARASCO.

2.5 BEHAVIORAL OUTCOMES. Longer term neurological or behavioral outcomes have not been measured in controlled, randomized trials with DHA/ARA supplemented *vs.* unsupplemented formula-fed babies. However, there have been several studies looking at the long term outcomes of infants who were breast-fed (thereby receiving nutritional DHA and ARA early in life) compared to those who were formula-fed (deficient in DHA and ARA early in life). Lanting *et. al.* (155) have demonstrated that after 9 years, there was a significantly higher frequency of minor neurological dysfunction in children who were formula-fed compared to those who were breast-fed. Similarly, Stevens *et. al.* (227) demonstrated that formula-fed infants had a significantly higher risk of developing attention deficit hyperactivity disorder (ADHD) by 6-12 years of age. Indeed, those children who displayed the greatest degree of ADHD also had the lowest DHA status as measured by circulating levels of DHA.

Additional studies with non-human primates further support the role of DHA and ARA in infant development. Baby monkeys that were fed an infant formula without supplemental DHA and ARA performed more poorly on various motor skill and visual orientation tests compared to babies who were fed a formula supplemented with DHASCO and ARASCO (54). Jensen *et. al.* (140) have also recently reported an improvement in the motor development (Gesell Gross Motor Development Quotients) in human infants who were breast-fed by mothers receiving DHASCO as a dietary supplement (this significantly elevates the DHA levels in their breast milk), compared to infants who were breast-fed by mothers receiving a placebo.

2.6 GROWTH/MORBIDITY/MORTALITY OUTCOMES. A recent study by Diersen-Schade *et. al.* (77) demonstrated that DHASCO and ARASCO-fortified preterm infant formulas enhanced the growth of preterm infants compared to standard, unsupplemented preterm formulas (Figure 2.5-1). Not only was the growth rate enhanced, but by 57 weeks postmenstrual age, the DHASCO/ARASCO-supplemented formula fed preterm infants also attained a weight comparable to a human milk-fed full term infant. Another study with term infants fed DHA/ARA-supplemented formula similarly found improved growth in the supplemented infants compared to the standard formula controls (41).

To the best of our knowledge, only one study has investigated the effect of DHA/ARA supplemented formulas on one of the most serious issues of morbidity and mortality affecting preterm infants. Carlson *et. al.* (42) recently reported that there was a highly significant 83% reduction in the incidence of necrotizing enterocolitis (NEC) in preterm infants who were fed formulas supplemented with DHA/ARA (using egg yolk lipids) compared to those receiving standard preterm formulas. Preterm infants who receive breast milk also have a significantly lower incidence of NEC compared to formula-

fed infants. In other words, preterm infants who are receiving any form of enteral nutrition that does not contain DHA and ARA (*i.e.*, all formulas in the U.S. today) are at a much higher risk for NEC than otherwise. These data were consistent with an animal (rat) model of NEC established by Caplan and colleagues (38). In this case, animals supplemented with DHASCO and ARASCO in a dietary formula exhibited a similar significant reduction of NEC when stressed compared to pair matched animals receiving a standard formulation.

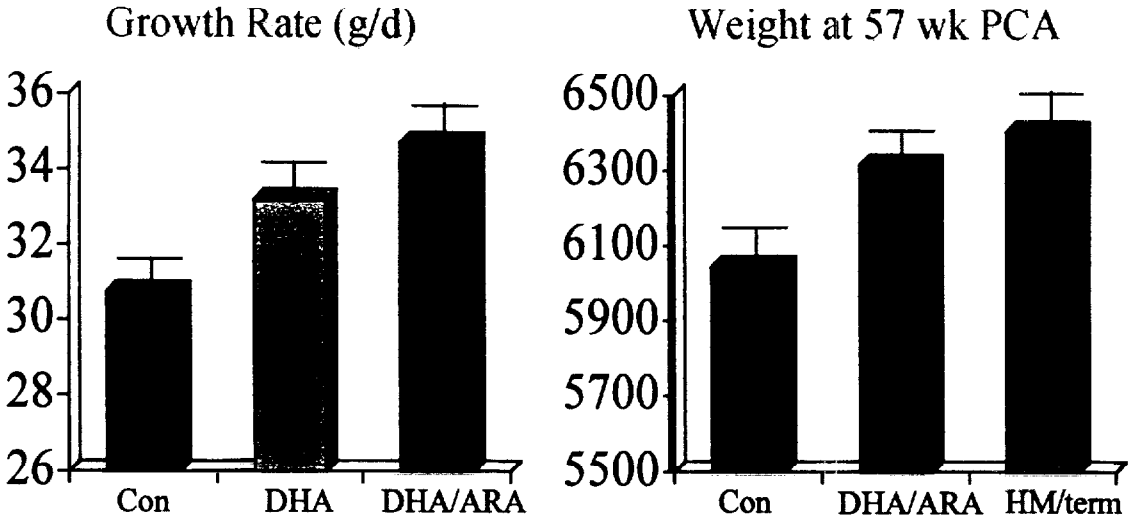

Figure 2.5-1. Growth rates and attained weights at 57 weeks postconceptual age (PCA) of preterm infants fed formulas enriched with DHASCO alone, or a combination of DHASCO and ARASCO. The attained weight of a full term infant fed human milk is shown for comparison. Data redrawn from Hansen et al (112).

2.7 CHRONIC LONG-TERM OUTCOMES. Infants who are breast-fed have been shown to have higher DHA levels in red muscle compared to infants who are formula-fed (12). The breast-fed infants also had a lower fasting glucose level compared to formula-fed infants, and a correlation between higher skeletal muscle DHA and lower fasting glucose was established by these researchers. This correlation was also present within the normal variability of skeletal DHA levels in the formula-fed group alone. This recent observation is particularly relevant because skeletal muscle is the major site of insulin-mediated glucose uptake in the body, and insulin resistance (indicated by high fasting glucose levels) is associated with such diseases as non-insulin dependent diabetes mellitus, obesity, dyslipidemia, hypertension and heart disease. Consistent with this finding was a recent report that breast-fed infants have a significantly lower risk of obesity later in life (risk odds ratio 0.75) than formula-fed infants (82, 243).

Schizophrenia may in some cases be a neurodevelopmental disorder and in a recent study by McCreadie (172), mothers of 45 schizophrenic patients in Nithsdale, southwest

Scotland, were asked to complete a questionnaire about whether or not their offspring had been breast-fed. Those patients who had not been breast-fed had significantly more schizoid and schizotypal personality traits in childhood and a poorer social adjustment than their sibs. Breast-fed patients did not differ from their sibs. The author then concluded that since fewer patients than normal were breast-fed, the lack of breast milk may be a risk factor in the neurodevelopmental form of schizophrenia.

Finally, a new retrospective study looking at 2,200 children with either acute lymphoblastic or acute myeloid leukemia in comparison to 2,418 pair-matched controls, concluded that breastfeeding was associated with a highly significant 21% reduction in the risk of acute childhood leukemias (183). Furthermore, the authors concluded that the risk reductions were greatest for children breastfed more than 6 months. Since breastfeeding is the normal situation, an alternative conclusion would be that formula feeding resulted in a highly significant increase in the risk of acute childhood leukemias. Although this study did not assess the DHA status of the patients or controls, based on previous work it is likely that those children who were formula-fed had a lower DHA status that those children who were breast-fed as infants. This observation is particularly interesting in light of some recent cancer work in animal models by Rose and colleagues (201). They showed that animals with a higher DHA status (provided by the dietary supplementation with DHASCO) were more resistant to induced cancers and, once a cancer was established, they had a slower rate of metastasis than unsupplemented animals.

2.8 REPORTS INCONSISTENT WITH GRAS STATUS OF DHA AND ARA FROM OTHER SOURCES. We are not aware of any reports that are inconsistent with the GRAS status of ARASCO and DHASCO for use in infants, children or adults. However, there have been three reports in the literature noting potential negative findings in human infants when fed an infant formula using fish oil as a source of DHA. It is important to note that all fish oils contain EPA, a fatty acid expressly absent (by design) from DHASCO, and did not contain supplemental arachidonic acid. Although these findings involve materials that are not the subject of this GRAS affirmation, these reports are reviewed below.

Effects on Growth. In one of the first clinical studies in which DHA supplementation was tested, a fish oil rich in EPA was used as a DHA source (0.3% EPA and 0.2% DHA). This formula was provided to preterm infants through nine months past their term date (48). No supplemental ARA was provided. Under these conditions, Carlson and co-workers reported an even greater decline in serum ARA compared to standard formulas, and they measured a reduced growth rate in those infants. Upon further analysis, it became clear that there was a correlation between the growth of the infants and their ARA status (47). Since EPA is a well-known antagonist of ARA metabolism, it was not surprising that providing an EPA-rich fish oil to an infant would further compromise the infant's ARA status which was already low because of the lack of dietary ARA in the formula. Subsequent studies indicated that ARA may affect infant growth through ARA-derived eicosanoids that elevate the levels of growth hormone (18). Two subsequent clinical studies (39, 203) which both used fish oil with a lower EPA content (formulas contained 0.04 - 0.07 wt % EPA and 0.2 wt % DHA) but no

supplemental ARA, also reported reduced growth, albeit less pronounced than the earlier Carlson study. In the case of Ryan and coworkers (203), the effect was limited to boys. A more recent study with term infants using fish oil supplemented formulas showed no effect on growth (167). In several large, well-controlled, multicenter trials using DHASCO as a source of DHA in the formulas (there is no EPA in DHASCO), there was no inhibition of growth, even in groups where DHASCO was used without any additional ARA (see Appendix 2 for details). In fact, two studies have now reported that the combination of DHASCO and ARASCO actually results in improved growth of preterm (77) as well as full term infants (41) compared to infants fed control formulas. These reports suggest that EPA at any level is an undesirable component of infant formulas. This fact was also recognized by a recent expert workshop in Washington DC which not only recommended the addition of DHA and ARA to infant formulas, but established an upper limit (not to exceed) for EPA in infant formulas (217). One of the main reasons that DHASCO has been chosen over fish oil as a source of DHA in infant formulas sold outside of the United States is that DHASCO contains no EPA.

Language Development in Infants. In another major study, language development at 14 months of age was reported to have been negatively affected in the group receiving fish oil-supplemented formula (212). The authors subsequently reported that this difference was not present at three years of age (213). Furthermore, this negative effect was only associated with the fish oil (EPA-containing) formula without supplemental ARA since no such effect was observed in a parallel arm using a formula containing both DHA and ARA. In fact, there was a three-point improvement (not statistically significant) in the developmental quotients (DQ) of the DHA/ARA supplemented children (213). There have been no similar negative reports when DHASCO/ARASCO supplemented formulas have been used. In fact, it has recently been reported that at 18 months of age there is a seven point improvement in the DQ of infants who received DHASCO/ARASCO supplemented formulas compared to those receiving standard formulas as assessed by the Bayley infant development index (21). Vocalization and language skills are important components of this test.

SIDS. The aforementioned trial which noted reduced growth in boys fed formulas supplemented with an EPA-containing fish oil, also reported more SIDS-related deaths in the group fed the fish oil (EPA-containing) supplemented formula with no ARA, than in the control group (203). An independent safety committee, which included medical specialists with expertise in SIDS, carefully reviewed each case and concluded that none of the SIDS deaths were related to the dietary treatments. In support of this opinion, none of the other trials (more than 30 to date involving over 2,500 babies) where DHA and ARA supplemented formulas have been used, regardless of the sources, have noted any similar observation. Breastfeeding, which supplies DHA and ARA to the infant is associated with a reduced risk of SIDS (90, 178), not an increased risk. Furthermore, there have been no reports of frequencies of SIDS changing in countries where DHA and ARA supplemented formulas have been in extensive use for several years (*e.g.*, Israel), and no reports of such adverse events in postmarket surveillance after launching

DHASCO/ARASCO supplemented formulas (*cf.* Wyeth Ayerst Research, personal communication).

Bleeding Times. Although there have been no reports of excessive bleeding in controlled infant formula trials using fish oil-supplemented formulas, there are observations in children and adults of increased bleeding times when consuming fish oils (30, 218, 251). This, again, is entirely consistent with an effect of the eicosanoid precursor – EPA – contained in fish oil, downregulating the omega-6 eicosanoids PGE2 and TXA2, both of which are important in stopping bleeding in response to an injury. Since this is an EPA-mediated response, and neither DHASCO nor ARASCO contain EPA, this issue does not apply to these products. However, bleeding times were tested in a well-controlled, USDA-sponsored study in adults maintained in a metabolic ward and fed 15 g of DHASCO every day for a period of 90 days. At the end of the trial, as expected, there were no changes in platelet aggregation or prothrombin bleeding time in the DHASCO-treated subjects compared to the placebo controlled group (187).

2.9 RECOMMENDATIONS OF OTHER EXPERT PANELS. Several other Expert Panels have been convened over the last 10 years to assess the issue of whether or not infant formulas should contain long chain polyunsaturated fatty acids (*i.e.*, DHA and ARA). There has been general agreement among all of them regarding the importance of DHA and ARA in infant nutrition, but each panel approached the issue with different questions or objectives in mind. Such questions ranged from the allowance of such supplements to their requirement in all infant formulas. The most recent Expert Panel actually established what it considered to be Adequate Intake (AI) levels for DHA and ARA (*i.e.*, those levels required to support normal brain growth and development in infants). The various Panels' recommendations are listed in chronological order below.

1991. European Society of Pediatric Gastroenterology and Nutrition (ESPGAN). In an ESPGAN Committee Report commenting on the content and composition of lipids in infant formulas (83) the importance of long chain polyunsaturated fatty acids (LCP) in infant nutrition was recognized. It noted that both preterm and term infants fed standard formulas *"develop LCP-depletion of structural lipids"* and that animal studies showed that prolonged omega-3 deficiency "*reduces DHA content in the brain and retina and impairs development of visual acuity, and possibly also of discrimination learning*". They concluded that "*during this period of life, LCP are therefore considered essential nutrients that should be supplied with the diet.*" The Committee felt that "*enrichment* [of low birth weight infant formulas] *with metabolites of both linoleic acid* [*i.e.*, ARA] *and alpha linolenic acid* [*i.e.*, DHA] *approximating levels typical of human milk lipids (n-6 LCP* [ARA] *1%, n-3 LCP* [DHA] *0.5% of total fatty acids) is desirable*". They went on to say that *"LCP supplementation to* [term] *infant...might also be of advantage, but further data on this question are required prior to a definite recommendation*". Over the intervening years, such data have been generated.

1992. British Nutrition Foundation. In 1992, a Task Force of Experts who were invited by the Council of the British Nutrition Foundation to review the state of knowledge make recommendations on the nutritional and physiological significance of unsaturated fatty acids (31). One section of that report, related specifically to unsaturated fatty acids in early development, recognized the importance of the delivery of DHA and ARA to the growing infant *in utero* by transplacental transfer and, during nursing, via breast milk. Much of this recognition was based on "*evidence from animal studies, which suggested that retinal function and learning ability are permanently impaired if there is a failure in the accumulation of sufficient DHA during development*". They concluded with recommendations that "*it would be prudent for DHA to be present in all infant formulae at the same level as it is present in human milk (i.e., 0.2% of total energy)*". This corresponds to about 20 mg DHA/kg/day or about 50 mg DHASCO/kg/day. Furthermore, the Task Force recommended "*that preterm infant formulae should contain DHA at a level of 20 mg DHA/kg/day*". Finally, the Task Force also recommended "*that infant formulae should contain preformed arachidonic acid in an attempt to replicate the fatty acid profile of human milk*".

1994. World Health Organization/Food and Agriculture Organization. In October of 1993, an Expert Consultation was established jointly between the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to review the latest scientific evidence and make appropriate recommendations on the role of dietary fats in human nutrition in order to assist policy makers, health care professionals, the food industry, and consumers. The report, published the following year (84), includes a chapter on lipids in early development where they note that in addition to rodent studies, "*studies with nonhuman primates confirm that n-3 deficiency depresses the development of retinal function and visual acuity*". Throughout the discussion they underscore the need to maintain adequate DHA and ARA supplies to the infant, from the preconceptual preparation of the mother to the post-partum nursing of the infant. The Expert Consultation's recommendation was that "*preterm formula should provide 60 mg of ARA, and 40 mg of DHA per kg body weight per day*". For term infants, the recommendation was for "*40 mg of ARA and 20 mg of DHA per kg per day*". This latter recommendation would correspond to 100 mg ARASCO/kg/day and 50 mg DHASCO/kg/day. The Consultation goes on to say that "*these levels were suggested to provide the greatest possible release of the full genetic potential of neural and visual development of the infants*".

1994. International Society for the Study of Fatty Acids and Lipids (ISSFAL). In June of 1994, a subcommittee of the Board of ISSFAL approved a series of recommendations for the essential fatty acid requirements for infant formulas. These followed the general tenet that "*human milk is the best and only proven source of fat and essential fatty acids in the infant diet*". ISSFAL recognized that "*preterm infants fed soy oil supplemented formulae have altered electroretinograms and delayed visual maturation relative to those fed DHA supplemented formulas or human milk. These changes are correlated to biochemical indices of low DHA status*". The report goes on to recommend that "*formulas for these* [preterm] *infants should provide 60-100*

mg/kg/day as preformed arachidonic acid", and "*should provide 35-75 mg...per kg body weight per day as DHA*". Although these recommendations were detailed for preterm infants, the statement goes on to acknowledge that "*In general, the specific recommendations given above are applicable to term infants; however, the requirements for long chain polyunsaturates (AA and DHA) for term infants await the results of clinical trials that are now in progress*". Those studies have now been completed and they resulted in the new recommendations (see below) issued five years later in June of 1999 (217).

1998. Life Sciences Research Organization (LSRO). In September of 1995, under contract with the FDA, the LSRO began to evaluate the evidentiary basis for the requirement of various components in infant formulas for healthy term (but not preterm) infants. Among other issues, the requirements for DHA and ARA by full term infants was reviewed. Throughout the discussion of this issue, the Panel repeatedly recognized the need to maintain an adequate DHA and ARA status in the infants. Although the Panel considered the evidence available to it to be "*insufficient to warrant a recommendation that DHA and ARA be added to full term infant formulas at this time*", it did recognize that the relevant clinical trials were ongoing and that this issue should be readdressed in the near future. The LSRO Panel based its opinion on only four (9, 22, 40, 163, 166) of the ten term infant intervention studies listed in Table 2-2.1 where DHA/ARA supplemented formulas were used and functional outcomes were measured. The Panel's conclusion was still somewhat surprising since three of the four studies considered (9, 22, 40, 163, 166) clearly demonstrated a deficit in visual and/or neurological parameters, caused by standard formula feeding relative to human milk, that were overcome in all cases by the supplementation of DHA and ARA to the formulas. The fourth study (9) showed no difference in visual or neurological assessments between human milk-fed, standard formula-fed, and DHA/ARA supplemented formula-fed infants, but this study has been criticized due to the fact that the DHA level in the human milk was very low, as was the enrichment level in the formula (0.12%). These levels would deliver only about 6-10 mg DHA/kg body wt/day to the infant (about one-third that recommended by the WHO/FAO Expert Panel). The Panel also suggested that the DHA status of the infant could be improved if enough precursor alpha linolenic acid (ALA) was added to the formulas, and therefore recommended that all term formulas contain a minimum of 1.75% of energy as ALA. "*This recommendation was based on the essentially of ALA as a precursor of the n-3 series of LCPUFAs*" (*i.e.*, DHA), underscoring the importance of maintaining an adequate DHA status. Interestingly however, human milk contains very little ALA and the Panel made the unprecedented recommendation that formula should contain at least twice as much ALA as is found in breast milk (141). The recommendation is inconsistent with the intent of the Infant Formula Act, which was to have infant formula more closely match the gold standard of human breast milk. Furthermore, the Panel recognized that babies fed formulas with ALA contents even as high as 3.2% of total fatty acids still did not achieve the DHA status of breast-fed babies (139). Despite their lack of a recommendation for the requirement of DHA and ARA in all term formulas, "*the Expert Panel strongly endorsed breast feeding as the preferred source of nutrients for infants and, for a number of nutrients* [other than

DHA and ARA for some reason], *used the amounts found in breast milk as a guide in establishing minimum and maximum levels*". The Panel also recognized that the "*levels of DHA in the breast milk of women consuming a typical North American diet are generally found to be in the range of 0.2% - 0.4% of fatty acids*", but levels as high as 1.4% have been reported elsewhere (126). These typical breast milk levels would provide about 10 to 25 mg DHA/kg body wt/day for an infant.

1998 Health Canada. A working group assembled by Health Canada concluded that commercial formulas in Canada contain adequate amounts of linoleic and linolenic acids (37). However, they also recognized that *"it may be that ARA and DHA synthesis* [from these precursors] *is too slow to meet the tissue needs* [of the infant]". They further stated that *"clinical studies have attempted to determine the need for a dietary source or for the addition to infant formulas of ARA and DHA, but have yielded inconsistent results*". However, they cite only two of the ten available studies (see Table 2-2.1) which analyzed functional outcomes. Finally, they did agree that "*it is reasonable to presume that infants not fed breast milk might benefit from dietary sources of ARA and DHA*", but they did not recommend their addition until the safety of the sources of these fatty acids, not the fatty acids themselves, could be established.

1999. ISSFAL Workshop on the Essentially of and Recommended Dietary Intakes for Omega-6 and Omega-3 Fatty Acids. In April of 1999, an International workshop sponsored by NIH, The Center for Genetics, Nutrition and Health, and ISSFAL, and co-sponsored by several industry groups, was convened to make recommendations to the Board of Nutrition of the US National Academy of Sciences regarding the establishment of Dietary Reference Intakes (DRIs) for omega-6 and omega-3 intake (217). This group thought it was of "*utmost importance*" to focus on the composition of infant formula and recommended that an adequate intake (AI) for an infant formula diet would include 0.50% of total fatty acids as ARA and 0.35% of total fatty acids as DHA. A typical fat content of an infant formula of 5.3 g/100 kcal would lead to a consumption of about 5.8 g fat/kg body wt/day. This corresponds to about 30 mg ARA/kg body wt/day and 20 mg DHA/kg body wt/day; values which closely approximate the WHO/FAO Expert Panel recommendations of 1995. The ISSFAL Workshop also recognized the need for additional DHA supplementation to the mother during the period of pregnancy and lactation so that adequate delivery of DHA to the infant can take place from the maternal source. Consequently, they also recommended that "*during pregnancy and lactation women must ensure a DHA intake of 300 mg/day*".

2.10 CONCLUSIONS. A number of scientific and medical expert panels have critically discussed and evaluated the scientific evidence and concluded that the biochemical deficiency observed in the formula-fed infants can be eliminated by providing a source of DHA and ARA for those infants. The FDA has raised some safety concerns over the use of DHA and ARA in infant formulas (Appendix 4) and have indicated that more information was required before GRAS status can be applied for this use. These concerns have been addressed in this document and the Panel believes that they have been resolved in a dispositive fashion with scientific evidence. Furthermore, well controlled

clinical studies indicate that supplementing formulas with DHASCO and ARASCO reduces or eliminates the detrimental consequences of the DHA and ARA deficiency. Consequently, this Panel does not find any reason to disagree with other Expert Panels' opinions on the importance of DHA and ARA in infant nutrition, and further believes that there is now sufficient evidence that it is beneficial to supplement infants with DHA and ARA as a means to eliminate the biochemical deficiency in these infants and to more closely approximate the condition of a breast-fed infant. The Panel further believes that such an addition is in line with the general tenet of providing infants with nutritive components that most closely match the nutritive components of human milk.

3. CURRENT AND PROPOSED USES FOR DHASCO AND ARASCO

As described in the previous section, several other Expert Panels have addressed the issue of use levels of DHA and ARA in infant formulas (31, 83, 84, 133). The levels recommended by those Expert Panels are given in Table 3-1 for pre-term and term infant formulas and range from 0.4% to 1.5% of total formula fat for ARA, and 0.35% to 1.1% of total formula fat for DHA. These values closely approximate levels of DHA and ARA found in human milks from around the world. Although the recent LSRO Panel in the U.S. (159) concluded that there is not enough information at this time to mandate the addition of DHA and ARA to infant formulas (see previous discussion), they did conclude that there is ongoing research in this area, and that the issue should be reopened when those data are available. A much larger group of world-wide experts in lipid nutrition more recently (April, 1999) reviewed the latest data (not reviewed or discussed by the LSRO Panel), and concluded that an infant formula would need to contain at least 0.35% of lipid as DHA and 0.50% of lipid as ARA for an infant to receive an adequate intake (AI) of these components (217). They also went on to identify the potential problems with EPA and recommended that there should be an upper limit (not to exceed) for EPA of not more than one quarter the level of DHA.

Table 3-1. Recommendations of five expert panels for the inclusion of ARA and DHA in infant formulas.

	ESPGAN	*BNF*	*ISSFAL*	*FAO/WHO*	*ISSFAL*
Year	1991	1992	1994	1994	1999
Preterm Formula					
ARA (% of fat)[5]	1%	0.40 %	0.90 -1.5 %	0.90 %	0.50 %
DHA (% of fat)	0.5%	0.40 %	0.5-1.1 %	0.60 %	0.35 %
Term Formula					
ARA (% of fat)		0.40 %		0.70 %	0.50 %
DHA (% of fat)		0.40 %		0.35 %	0.35 %
DHA/EPA ratio			> 5 to 1	10 to 1	> 4 to 1

[5] Assmptions for the calculated values are 1) preterm infants consume 120 kcal/kg/d and term infants consume 110 kcal/kg/d, 2) fat comprises 50% of energy of the formula, and 3) 1 g fat has 9 kcal.

In the last 15 years, at least 32 well-controlled clinical trials have been completed using DHA/ARA supplemented formulas (see Table 2.2-1). All trials have used supplemental DHA at levels in preterm formulas from 0.15% to 0.78%, and for term formulas from 0.1% to 0.36% of total formula fat. Supplemental ARA has been used in most, but not all trials. In trials where supplemental ARA has been used, the concentration ranged from 0.1% to 1.1% of total fat in preterm formulas, and from 0.20% to 0.72% for term formulas. Of particular relevance to this Panel were the 14 clinical studies in which DHASCO and ARASCO were used as the DHA and ARA sources for supplementation (see Appendix 2). In those trials, the DHA and ARA dose levels ranged from 0.24 % to 0.76 % of total formula fat for DHA and 0.24 % to 1.1 % of total formula fat for ARA in preterm formulas. In full term infant formula trials, dose levels ranged from 0.20 % to 0.36 % of total formula fat for DHA, and from 0.20 % to 0.72 % of total formula fat for ARA. These values clearly fall well within the normal range of mother's milk (Figure 2.1-1) and the Expert recommendations (Table 3-1).

In the past five years, commercial infant formulas containing supplemental DHA and ARA have been introduced around the world. Of particular interest to this Panel were the formulas containing DHASCO and ARASCO as the forms of supplementation (Table 3-2). The Panel also noted that the LBW formula produced by Wyeth/Ayerst is provided to low birth weight infants under strict doctor's supervision. The manufacturer estimates that over 100,000 infants have been given these formulas under doctor's guidance. In fact, DHASCO/ARASCO-supplemented formulas are used more extensively around the world than formulas supplemented with any other source of DHA and ARA. The DHA levels in current commercial use for those formulas are 0.30% to 0.40% of formula fat for preterm formulas and from 0.25% to 0.40% of formula fat for term formulas. The ARA levels in current commercial use for those formulas are 0.45% to 0.60% of formula fat for preterm formulas and from 0.45% to 0.50% of formula fat for term formulas.

Table 3-2. Commercial infant formulas containing DHASCO and ARASCO

Country	*Company*	*Product Name*	*Term*	*Preterm*
Argentina	Wyeth	SMA LBW		X
Australia	Wyeth	SMA LBW		X
		SMA Gold	X	
	Numico	Nenatal		X
		KariCare First[6]	X	
Bahrain	Wyeth	SMA LBW		X
Bolivia	Wyeth	SMA LBW		X
Belgium	Numico	Nenatal		X
		Premilon		X
Chile	Wyeth	SMA LBW		X
China (P.R.C.)	Wyeth	SMA LBW		X
Colombia	Wyeth	SMA LBW		X
Cyprus	Wyeth	SMA LBW		X
Dominica	Wyeth	SMA LBW		X
Ecuador	Wyeth	SMA LBW		X
Egypt	Wyeth	SMA LBW		X

[6] KariCare First contains fish oil and ARASCO oil.

Country	Company	Product Name	Term	Preterm
El Salvador	Wyeth	SMA LBW		X
Finland	Numico	Nenatal		X
France	Wyeth	Modilac LBW		X
Greece	Wyeth	SMA LBW		X
Guatemala	Wyeth	SMA LBW		X
Guyana	Wyeth	SMA LBW		X
Haiti	Wyeth	SMA LBW		X
Honduras	Wyeth	SMA LBW		X
Hong Kong	Wyeth	SMA LBW		X
		SMA Gold	X	
Hungary	Wyeth	SMA LBW		X
Iceland	Wyeth	SMA LBW		X
Indonesia	Wyeth	SMA LBW		X
Ireland	Wyeth	SMA LBW		X
Israel	Maabarot	Materna Stage One	X	
Jamaica	Wyeth	SMA LBW		X
Jordan	Wyeth	SMA LBW		X
Kenya	Wyeth	SMA LBW		X
Kuwait	Wyeth	SMA Gold	X	
		SMA LBW		X
Lebanon	Wyeth	SMA LBW		X
Malawi	Wyeth	SMA LBW		X
Malaysia	Wyeth	SMA LBW		X
Malta	Wyeth	SMA LBW		X
Mauritius	Wyeth	SMA LBW		X
Mexico	Wyeth	SMA LBW		X
Netherlands	Numico	Nenatal		X
New Zealand	Wyeth	SMA LBW		X
		SMA Gold	X	
	Numico	Nenatal		X
		KariCare First	X	
Nicaragua	Wyeth	SMA LBW		X
Oman	Wyeth	SMA LBW		X
Panama	Wyeth	SMA LBW		X
Peru	Wyeth	SMA LBW		X
Philippines	Wyeth	SMA LBW		X
Portugal	Wyeth	SMA LBW		X
Qatar	Wyeth	SMA LBW		X
Russia	Wyeth	SMA LBW		X
Saudi Arabia	Wyeth	SMA LBW		X
Seychelles	Wyeth	SMA LBW		X
Singapore	Wyeth	SMA LBW		X
South Africa	Wyeth	SMA LBW		X
Spain	Novartis	Adapta PEG	X	
		Preadapta		X
	Numico	Nenatal		X
Switzerland	Numico	Preadapta		X
Taiwan	Wyeth	SMA LBW		X
Thailand	Wyeth	SMA LBW		X
Trinidad	Wyeth	SMA LBW		X
Turkey	Wyeth	SMA LBW		X
U.A.E.	Wyeth	SMA LBW		X

Country	Company	Product Name	Term	Preterm
		SMA Gold	X	
United Kingdom	Wyeth	SMA LBW		X
Uruguay	Wyeth	SMA LBW		X
Venezuela	Wyeth	SMA LBW		X
Vietnam	Wyeth	SMA LBW		X
Zimbabwe	Wyeth	SMA LBW		X

This Panel was asked by Martek Biosciences Corporation to review the published literature, the published and unpublished safety data, laboratory records and assessments of clinical and laboratory personnel, to make an assessment as to whether DHASCO and ARASCO can be considered as Generally Recognized as Safe when provided to both preterm and full term infants and children at doses for DHA corresponding to levels up to 1.0 % of daily fat intake, and for ARA corresponding to levels of up to 1.0 % of daily fat intake. This would correspond to 2.5% of daily fat intake as DHASCO and 2.5% of daily fat intake as ARASCO. Assuming human infants consume about 100 to 120 kcal/kg body wt/day, of which fat comprises about 50%, an infant will consume about 50 - 60 kcal/kg body wt/day of fat, or about 5.5 - 6.7 g of fat/kg body wt/day (1 g fat = 9 kcal). The above referenced DHASCO intake of 2.5% of daily fat for an infant would correspond to about 150 mg DHASCO/kg/day. The above referenced ARASCO intake of 2.5% of daily fat for an infant would also correspond to about 150 mg ARASCO/kg/day. These GRAS values are approximately two- to four-fold higher than levels presently used in LCP-supplemented commercial infant formulas (see Table 3-2).

4. IDENTITY AND DESCRIPTION

ARASCO and DHASCO are macronutrient triglyceride oils produced in fungal and algal microorganisms respectively. These microorganisms have not been genetically engineered. They are grown in axenic liquid culture, harvested, dried and the oils are extracted and processed using current Good Manufacturing Practices (cGMP). All processing materials are of food grade quality or better. These oils are approximately 40-55% enriched in arachidonic acid or docosahexaenoic acid and, consequently, represent a concentrated, well-defined source of these particular long chain polyunsaturated fatty acids (LC-PUFAs). They are generally diluted with another vegetable oil (High Oleic Sunflower Oil – HOSO) to a standard 40% DHA or ARA content prior to being shipped worldwide for addition to infant formulas. The manufacturing processes for the oils are described in detail in Section 5. The comprehensive chemical descriptions of the oils are described below.

4.1 CHEMICAL COMPOSITION OF DHASCO. DHASCO oil is triglyceride preparation that is enriched to 40% by weight in DHA and is made up of a mixture of an oil extracted from the marine microalgae *Crypthecodinium cohnii* and a commercial food-grade of HOSO. It is a free flowing, yellow-orange oil, which is predominantly triglyceride (>95%) with some diglyceride and nonsaponifiable material (<5%) as is typical for all food-grade vegetable oils. The fatty acid compositions of DHASCO from four

typical commercial batches are shown in Table 4.1-1. Minor fatty acid components listed as "other" in Table 4.1-1 generally constitute about 1% of the total fatty acid composition. A recent report (238) indicated the presence of small amounts of C28:8n-3 in DHASCO oil, as well as fish oil. This fatty acid is the next expected omega-3 end product of the Sprecher pathway beyond DHA (161) and is one of the minor components (approximately 1%) of DHASCO and many fish oils. Compositional analyses of other components of the oils from the same batches are provided in Table 4.1-2. The levels of these latter components compare favorably with other commercial edible oils. In general, the residual extraction solvent is typically absent or undetectable (detection limit <0.3 ppm), there are no detectable cyclic fatty acids or trans fatty acids, pesticide residues (negative for a standard panel of 74 pesticides)[7], or heavy metals such as arsenic, mercury and lead (all below the 0.1 ppm detection limit).

Table 4.1-1. Fatty acid composition of DHASCO from four separate batches.

Lot Number	*"8-2-14"*	*"8-150*	*"8-106*	*"8-3-110*
Date	4/98	3/98	2/98	12/97
C 8:0	0.35	0.13	0.18	<0.10
C 10:0	1.24	0.87	0.99	1.66
C 12:0	5.91	5.09	5.93	7.16
C 14:0	16.82	15.43	17.52	17.38
C 16:0	13.95	12.92	13.99	14.97
C 16:1	1.62	1.49	1.23	1.48
C 18:0	0.45	0.64	0.55	0.36
C 18:1	13.32	17.69	14.34	13.33
C 18:2	0.40	0.74	0.51	0.31
C 20:0	0.14	0.10	0.11	0.10
C 22:0	0.13	0.18	0.15	0.11
C 22:5 n-3	<0.10	<0.10	<0.10	<0.10
C 22:6 DHA	44.78	43.57	43.93	41.85
C 24:0	0.11	<0.10	<0.10	<0.10
Others	0.42	1.02	0.46	1.25

The nonsaponifiable fraction of DHASCO is generally about 1.5% and is made up primarily of sterols. Two independent laboratories[8] have analyzed the sterols of this fraction and their results are presented in Table 4.1-3. These data agree with results previously published on the sterol fraction of *C. cohnii* (190, 195, 250). The principle component of these sterols is a 4-methyl sterol, dinosterol (Figure 4.1-4). 4-methyl sterols are found in the normal metabolic pathway of cholesterol biosynthesis in man

Table 4.1-2. Chemical composition of DHASCO from four separate batches

[7] Notice of a Claim for Exemption from Premarket Approval, submitted to the Office of Premarket Approval, Food and Drug Administration by Wyeth Ayerst Laboratories on August 27, 1998.

[8] Analyses performed by Professor J. Weete, Auburn University, Alabama and Dr. J. Volkman, CSIRO Division of Oceanography, Australia.

Lot Number		*78-2-147*	*78-150*	*78-106*	*78-3-110*
Date		4/98	3/98	2/98	12/97
Chemical Analysis	units				
DHA	area %	44.78	43.57	43.93	41.85
DHA	mg/g	414.2	407.3	414.7	386.4
EPA	area %	<0.1	<0.1	<0.1	<0.1
Free fatty acid	%	0.16	0.14	0.22	0.16
Peroxide value	meq/kg	<0.1	<0.1	0.24	<0.1
Volatiles	%	<0.01	<0.01	<0.01	<0.01
Nonsaponifiables	%	1.85	1.55	1.36	1.79
Insoluables	%	<0.01	<0.01	<0.01	<0.01
Trans fats	%	<1.0	<1.0	<1.0	<1.0
Elemental Analysis					
Arsenic	ppm	<0.5	<0.5	<0.5	<0.5
Cadmium	ppm	<0.1	<0.1	<0.1	<0.1
Chromium	ppm	<0.1	<0.1	<0.1	<0.1
Copper	ppm	<0.02	<0.02	<0.02	<0.02
Iron	ppm	<0.02	<0.02	<0.02	<0.02
Lead	ppm	<0.1	<0.1	<0.1	<0.1
Manganese	ppm	<0.01	<0.01	<0.01	<0.01
Mercury	ppm	<0.04	<0.04	<0.04	<0.04
Molybdenum	ppm	<0.05	<0.05	<0.05	<0.05
Nickel	ppm	<0.1	<0.1	<0.1	<0.1
Phosphorous	ppm	<1	1	<1	1
Silicon	ppm	135	41	18	41
Sulfur	ppm	29	18	80	74

(93, 116, 168) and have been identified in several other food sources, including fish and shellfish (196). There is no *a priori* reason to believe that these sterols have any significant biological activity, and they would be expected to feed into the normal cholesterol metabolic pathways in humans. It is also important to note that at the proposed GRAS level for DHASCO of 2.5% of daily fat intake, the contribution of the sterols from the algal oil to the diet is very small relative to other phytosterols found in infant formulas (from the vegetable oil) or cholesterol provided by human milk. A dose level of 150 mg DHASCO/kg body wt/day would correspond to less than 3 mg total algal sterols/kg body wt/day. A typical infant formula would deliver about 33.5 mg of phytosterols/kg body wt/day (6700 mg fat/kg body wt/day and 0.5% sterols), and human milk delivers about 400 mg cholesterol per 100 g fat (*ca.* 30 mg/kg body wt/day) to an infant (141). If mothers are consuming a phytosterol-rich diet, the amount of phytosterol in the breast milk can be as high as the cholesterol levels (174).

Table 4.1-3 Sterol content of DHASCO from three independent laboratories

					Percent of Total Sterols

Sterol Fraction		*Common Name*	*Volkman*[7]	*Weete*[8]	*Withers (250)*
4α,23,24-trimethyl cholesta-22-en-3β-ol	C30:1	dinosterol	31.5	25.5	40
cholesta-5,7-dien-3β-ol	C27:2	dehydrocholesterol	9.6[9]		14
4α,24-dimethyl cholestan-3β-ol	C29:0		9.2		minor
4α,23,24-trimethyl cholesta-5,22-dien-3β-ol	C30:2	dehydrodinosterol	8.2		major
cholesta-7-en-3β-ol	C27:1	lathosterol	7.5		minor
4α,24-dimethyl cholesta-22-en-3β-ol	C29:1		6.4	6.2[9]	
4α,23,24-trimethyl cholesta-22-en-3β-ol	C30:1	dinosterone	6.0	8.8[9]	14
23,24-dimethyl cholesta-5-en-3β-ol	C29:1		4.6		
4α,23,24-trimethyl cholesta-24(28)-ene-3β-ol	C30:1		4.2	3.6[9]	
cholesta-x,x-dien-3β-ol[10]	C27:2		3.6[8]	9.8	trace
*cholesta-5,24-dien-*3β-ol	C27:2	desmosterol	2.4[9]	9.4	
cholesta-5-en-3β-ol	C27:1	cholesterol	1.7[9]	2.7	2
23 or 24-methyl cholesta-5,7-dien-3β-ol	C28:2		1.9[9]		trace
	C27:3		1.3		
a 5,7-dien sterol	C29:2			6.9[9]	trace
Total sterols (mg per g dry weight)			9.5		30

Triglycerides are the predominant component of any natural fat or oil and are present as a family of compounds wherein the various fatty acids may be found attached to any of the three positions on the glycerol backbone (see Figure 2.1-1). Corn oil, for example, is made up of at least 21 individual triglycerides (228). In some cases, certain fatty acids are found preferentially in either the Sn-1, Sn-2 or Sn-3 positions on the glycerol backbone. In human milk, for example, palmitic acid is commonly found on the Sn-2 position, whereas in vegetable oils found in infant formulas, palmitic acid is almost never found on the Sn-2 position (129). Despite these discrepancies, the oils in infant formula are generally hydrolyzed and the fatty acids are taken up efficiently by the infant gut (see Section 7.1 for a description of absorption, distribution, metabolism and excretion of dietary fats and oils). Nevertheless, it would be desirable to match as closely as possible the positional placement of DHA in human milk triglycerides. We could find only two references relating to the positional specificity of DHA and ARA in human milk triglyceride and they indicate that about 50-60% of the DHA is found on the Sn-2 position (127, 170). Myher *et. al.* (182) have reported that approximately 45% of the DHA found in DHASCO is also located on the Sn-2 position. Since the triglyceride structure of DHASCO is quite similar to human milk with respect to the positional specificity of DHA, there would be no reason to believe that the digestion and absorption of DHA from DHASCO should be any different than the DHA from human milk fats. In fact, Carnielli *et al.* (51) have tested this and shown that the absorption of DHA from DHASCO in human infants more closely approximates that of human milk than if DHA is presented in the form of a phospholipid. Many clinical studies have also shown that blood levels of DHA increase to levels matching those of the breast-fed infant when infants are fed DHASCO-supplemented formulas (see Appendices 2 and 3).

[9] Assignment based on known number of carbons and double bonds as well as number and/or placement of methyl groups, all of which are consistent with the cited chemical structure.
[10] The x refers to unassigned double bond placement.

4.2 CHEMICAL COMPOSITION OF ARASCO. ARASCO oil is a macronutrient triglyceride preparation that is enriched to 40% by weight in ARA and is made up of a mixture of an oil extracted from the soil fungus *Mortierella alpina* and a commercial grade of HOSO. It is a free flowing, yellow oil, which is predominantly triglyceride (>95%) with some diglyceride and nonsaponifiable material (<5%) as is typical for food-grade vegetable oils. The fatty acid composition of ARASCO from four typical commercial batches are shown in Table 4.2-1. Compositional analyses of the other components of the oils from the same batches are provided in Table 4.2-2. These latter components are found at levels similar to those in other commercial edible oils. In general, the residual extraction solvent is typically absent or below detection (detection limit <0.3 ppm), and there are no detectable cyclic fatty acids or trans fatty acids, pesticide residues (negative for a standard panel of 74 pesticides;(88)), or heavy metals such as arsenic, mercury and lead (all below the 0.1 ppm detection limit).

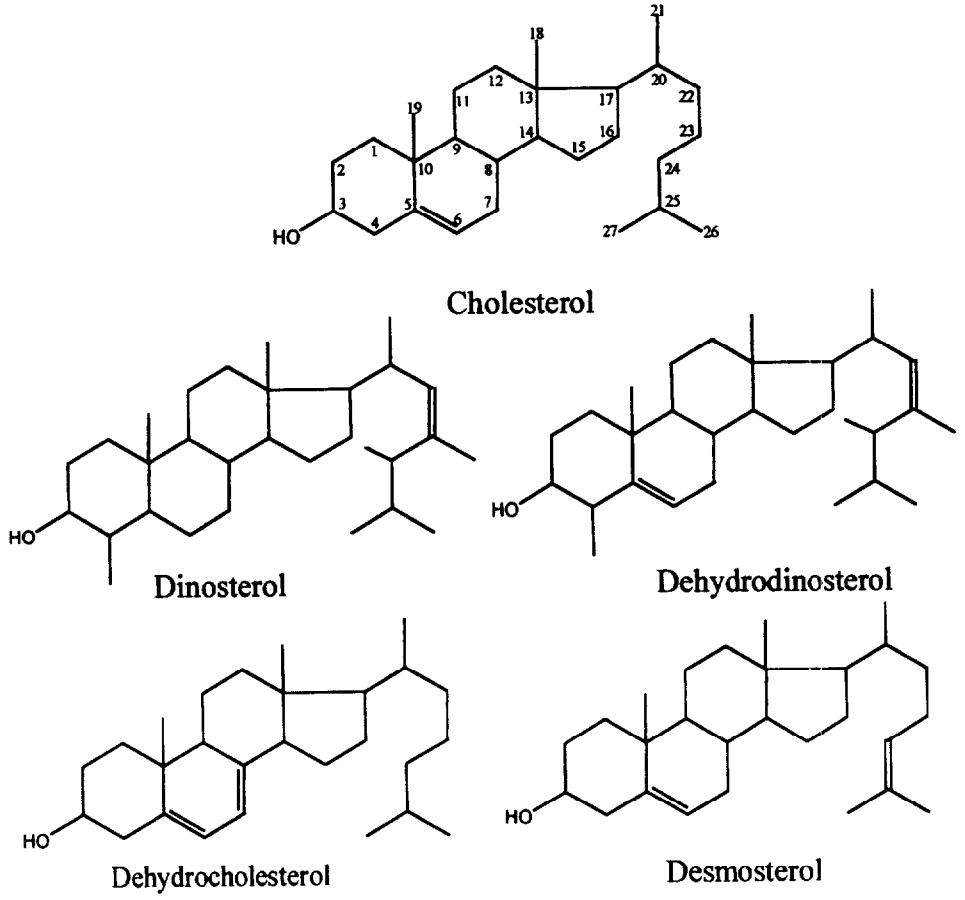

Figure 4.1-4 Structures of common sterols in DHASCO and ARASCO.

Table 4.2-1. Fatty acid composition of ARASCO from four separate batches.

Lot Number	*87-02-158*	*87-242*	*87-123*	*87-92*
Date	3/99	10/98	6/98	10/97

C 14:0	0.58	0.34	0.39	0.44
C 16:0	9.59	7.32	7.17	8.45
C 17:0	0.42	0.38	0.33	0.41
C 18:0	10.2	9.13	7.60	9.24
C 18:1 n-9	15.95	20.82	23.35	18.62
C 18:1 n-7	0.14	0.28	0.32	0.37
C 18:2 n-6	7.62	6.77	5.56	7.16
C 18:3 GLA	2.99	2.82	2.45	2.81
C 20:0	0.96	0.93	0.83	0.92
C 20:1 n-9	0.40	0.35	0.36	0.50
C 20:2 n-6	0.62	0.57	0.60	0.72
C 20:3 DGLA	2.57	2.03	1.82	1.43
C 20:4 ARA	42.69	42.96	44.26	43.14
C 22:0	2.02	2.02	1.98	2.00
C 24:0	1.92	1.91	2.04	1.85
C 24:1 n-9	0.11	0.13	0.23	0.20
Others	1.22	1.24	0.65	0.78

Table 4.2-2. Chemical composition of ARASCO from four separate batches.

Lot Number		*87-02-150*	*87-242*	*87-123*	*87-92*
Date		3/99	10/98	6/98	10/97
Chemical Analysis	units				
ARA	area %	42.69	42.96	44.26	43.14
ARA	mg/g	406.4	409.0	416.4	406.3
EPA	area %	<0.1	<0.1	0.16	<0.1
Free fatty acid	%	0.18	0.11	0.10	0.27
Peroxide value	meq/kg	1.2	0.41	0.12	1.51
Volatiles	%	<0.01	0.03	<0.01	0.02
Nonsaponifiables	%	1.73	1.36	1.69	1.18
Insoluables	%	<0.01	<0.01	<0.01	<0.01
Trans fats	%	<1.0	<1.0	<1.0	<1.0
Elemental Analysis					
Arsenic	ppm	<0.5	<0.5	<0.5	<0.5
Cadmium	ppm	<0.1	<0.1	<0.1	<0.1
Chromium	ppm	<0.1	<0.1	<0.1	<0.1
Copper	ppm	<0.02	<0.02	<0.02	<0.02
Iron	ppm	<0.02	<0.02	<0.02	<0.02
Lead	ppm	<0.1	<0.1	<0.1	<0.1
Manganese	ppm	<0.01	<0.01	<0.01	<0.01
Mercury	ppm	<0.04	<0.04	<0.04	<0.04
Molybdenum	ppm	<0.05	<0.05	<0.05	<0.05
Nickel	ppm	<0.1	<0.1	<0.1	<0.1
Phosphorous	ppm	<1	<1	<1	<1
Silicon	ppm	310	280	280	350
Sulfur	ppm	5	3	6	4

About 1.5% by weight of ARASCO is a nonsaponifiable fraction which is composed primarily of sterols. Two independent laboratories[11] have analyzed the sterols of this fraction and their results are presented in Table 4.2-3. These data are in agreement with published results on the sterol fraction of *M. alpina* (215). The principle component of these sterols is desmosterol (Figure 4.1-4). Desmosterol is found in the normal metabolic pathway of cholesterol biosynthesis in man (168) and is commonly found in several other food sources including animal fat, vegetable oils, and human milk (141). Shimizu (215) has reported the presence of a novel cyclopropyl-containing sterol, 24,25-methylenecholest-5-en beta-ol, in *M. alpina* at a level of 22% of the total sterols. This compound was not detected in the chromatograms of the Martek ARASCO samples by either of the two independent analytical laboratories. It is, therefore, possible that this compound was either misidentified by Shimizu, or that it was a real component unique to the strain 1S-4 developed by these Japanese workers. It is important to note that at the proposed GRAS level for ARASCO of 2.5% of daily fat intake, the contribution of the desmosterol from the fungal oil to the diet is very small relative to other phytosterols, cholesterol, and even desmosterol provided by human milk or infant formulas. At a dose level of 150 mg ARASCO/kg body wt/day, this would correspond to about 1 mg desmosterol/kg body wt/day. A typical infant formula would deliver about 33.5 mg of phytosterols/kg body wt/day (6700 mg fat/kg body wt/day and 0.5% sterols), and human milk delivers about 400 mg cholesterol per 100 g fat (*ca.* 30 mg/kg body wt/day) to an infant (141). Desmosterol is the second most abundant sterol in human milk (141) and is present at levels of about 10% that of cholesterol (*i.e.*, about 3-4 mg/kg body wt/day delivery to the baby). If mothers are consuming a phytosterol-rich diet, the amount of phytosterol in the breast milk can be as high as the cholesterol levels (174).

Table 4.2-3 Sterol content of ARASCO from three independent laboratories.

Sterol Fraction		*Common Name*	*Percent of Total of Sterols*		
			Volkman	*Weete'*	*Shimizu (215)*
cholesta-5,24-dien-3β-ol	C27:2	desmosterol	67.3	66.4	58
24-methyl cholesta-5,24(25 or 28)-dien-3β-ol	C28:2		14.0	13.4[12]	11
24-methyl cholesta-5,25-dien-3β-ol	C28:2		12.3[13]	9.7[14]	9
	C28:2		2.1	3.6	
4α,4β,14-trimethyl-8,24-dien-3β-ol	C30:2	lanosterol	1.1	2.7	
cholesta-5,25-dien-3β-ol	C27:2		2.0	1.0	
24,25-methylene cholesta-5-en-3β-ol	C28:1		nd	nd	22
Total sterols (mg per g)			7.9		5.3

[11] Analyses performed by Professor J. Weete, Auburn University, Alabama and Dr. J. Volkman, CSIRO Division of Oceanography, Australia.

[12] Identified as C28$\Delta^{5,x}$

[13] Identified as C28:2

[14] Identified as C28$\Delta^{5,x}$

As discussed with DHASCO (see section 4.1), it would be most desirable for the ARA in ARASCO to have a positional placement on the triglyceride backbone that closely matches the position of this fatty acid in the triglycerides found in human milk. Two references have reported that about 45% of the ARA in human milk triglyceride is located on the Sn-2 position (127, 170). Myher *et. al.* (182) have reported that approximately 29% of the ARA found in ARASCO is also located on the Sn-2 position. Since the triglyceride structure of ARASCO is quite similar to human milk with respect to the positional specificity of ARA, there would be no reason to believe that the digestion and absorption of ARA from ARASCO should be any different than ARA from human milk fats. In fact, studies have demonstrated that blood levels of ARA increase to levels matching those of breast-fed babies when those infants are fed ARASCO-supplemented formula (see Appendix 2).

5. MANUFACTURING PROCESSES

5.1 DHASCO

5.1.1 NATURAL HISTORY OF *C. COHNII*. *Crypthecodinium cohnii* is a member of the Dinophyta (dinoflagellates). This is a distinct phylum of unicellular eucaryotic microalgae comprising an estimated 2,000 species (234). Morphologically, the cells are characterized by two flagella; one in a groove that encircles the middle of the cell and the other in a groove along the length of the cell. The nucleus lacks histone proteins, and the chromosomal DNA is permanently present in a condensed form (231).

Ecologically, the Dinophyta comprise an extremely important and vastly abundant group of primary producers in both freshwater and marine environments. Among the eukaryotic algae they are second only to the diatoms as primary producers in coastal waters (234). As such, they represent a primary link in the food chain of all marine and freshwater animals. In tropical and subtropical marine environments some species of Dinophyta have been found in a symbiotic relationship with many invertebrates, especially corals, and serve a critical role as the major primary producers in reef ecosystems (234). In these associations the host organism is able to swallow the alga and incorporate it into its own tissue without harming it. The algal cell then produces sugars, which are utilized by the host. Some organisms rely almost exclusively on the food provided by their endosymbiotic algal cells.

Most species of the Dinophyta are photosynthetic, but there are also several heterotrophic species (234). Heterotrophic Dinophyta feed on diatoms and other protists and, as a result, they can also be the dominant grazers in certain environments.

Like most unicellular organisms, the most common form of reproduction for the Dinophyta is asexual, where daughter cells form by simple mitosis and division of the parent cells (192). Under some conditions, sexual reproduction can occur and motile gametes, which are formed by mitosis, fuse to form a dormant zygote or resting cell. This zygote can have a very thick cell wall and is extremely resistant to hostile environmental

conditions. In fact, these thick-walled zygotes are well preserved in the fossil record and have indicated that this group of organisms is at least 400-600 million years old (234).

A small number of photosynthetic species of Dinophyta are known to produce a group of closely related toxins (226). These toxins are passed through the food chain by grazing organisms and can contaminate fish and bivalves. However, these toxin producing species are few in numbers, and there are no known heterotrophic species of the Dinophyta which are toxin producers. There are also no described cases of any of the Dinophyta species, or any other algae, being pathogenic to man or other mammals.

The genus *Crypthecodinium* is monospecfic (*i.e.*, containing a single species, *C. cohnii*). While containing the vegetative and nuclear characteristics in common with all other species of the Dinophyta, *C. cohnii* is further characterized by being incapable of photosynthesis. Thus, it is an obligate heterotroph. The natural ecology of this organism has been studied, and isolates *of C. cohnii* have been collected from all around the world (14). *C. cohnii* is frequently isolated from the surface of macroalgae (13). Because this species is easy to cultivate in the laboratory, it has become one of the best studied of all the algae, particularly with regard to ultrastructure, biochemistry and genetics.

Crypthecodinium cohnii, like other algae, reproduces primarily by asexual cell division (mitosis) and is haploid in the vegetative stage (13). This organism is capable of sexual reproduction in the laboratory, and with the exception of having a single division meiosis, has normal genetic behavior (13). Genetic studies indicate that *C. cohnii* exhibits genetic stability consistent with other eukaryotic species used in fermentative production of foods and food additives (13-15, 232); that is, there is very little genetic drift in these algae. In the many reports of *C. cohnii* in culture over the last 30 years, there has never been any indication that *C. cohnii* produces any toxin, nor is it related to any toxin-producing species (78). More broadly speaking, there are no known heterotrophic Dinophyta species, which are either toxigenic or pathogenic (226).

5.1.2 DHASCO FERMENTATION PROCESS. The diagram in Figure 5.1.2-1 pictorially describes the DHASCO Fermentation Process. The *C. cohnii* strain used for the production of DHASCO is proprietary to Martek (US Patents 5,397,591, 5,407,957 and 5,492,938) and the process has been published (152). The strain originated from the University of Texas culture collection and has been selected for rapid growth and high levels of production of the specific oil. The specific production strain of *C. cohnii* has been deposited with American Type Culture Collection (ATCC # 40750) under the obligations of the U.S. patent relating to its use. Master seed banks of the production strain are maintained under liquid nitrogen conditions at Martek and at ATCC. Working seed stock prepared from this master seed bank is also maintained cryogenically. On initiation of a production run, an individual ampoule from a working seed is used to inoculate a shake flask.

The medium used to grow *C. cohnii* from shake flask to production scale contains dextrose (Corn Products), yeast extract or a hydrolyzed vegetable protein (Universal Foods), sodium chloride, calcium chloride and magnesium sulfate. All ingredients are of food grade quality or better. The medium is prepared according to Standard Operating Procedures.

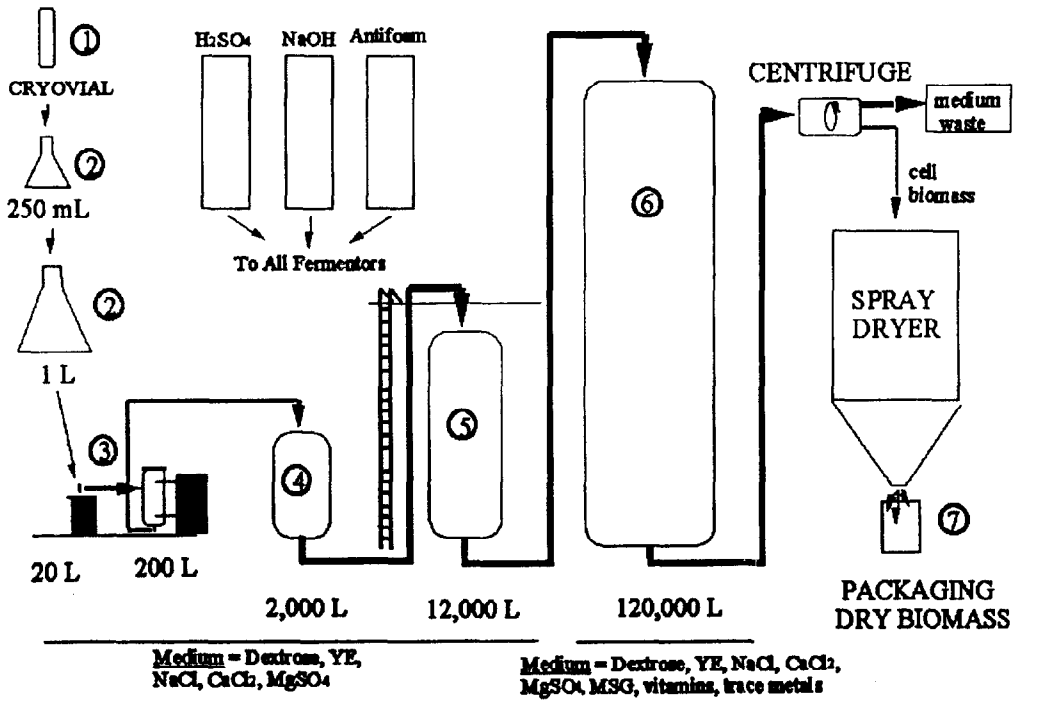

Figure 5.1.2-1. Flow chart of the stages of DHASCO fermentation scale-up.

Production scale fermentors use, in addition to the ingredients listed above, monosodium glutamate, potassium phosphate, potassium chloride, ferric chloride in citric acid, cupric sulfate, boric acid, manganese chloride, zinc sulfate, thiamine HCl and D-biotin. All production scale ingredients are also of food grade quality or better.

The cultures are transferred to successively larger vessels based on growth parameters. Dextrose concentration, temperature, pH, airflow, pressure, agitation and dissolved oxygen are monitored and controlled. When the culture reaches a determined maximum cell density and the fatty acid content reach a determined percentage, the culture is harvested by centrifugation and dried in a spray drier. The dried biomass is flushed with nitrogen and packaged in poly-lined boxes or Super Sacs prior to transport to the oil processing facility or frozen storage facility.

The quality of all raw materials used in fermentation scale-up is verified from the manufacturer's Certificate of Analysis and by Martek's policy of procuring ingredients only from verified reputable vendors of laboratory or food grade quality products. In order to maintain kosher certification, Martek must also ensure that ingredients are also procured from recognized suppliers of products meeting strict kosher guidelines. Martek products are certified kosher by the Orthodox Union (OU). The OU must approve deviations from the strictly controlled procurement source list prior to the introduction of an ingredient into the process from another supplier. This process has also been reviewed by the B.D.Z. Eda Charedith organization of Israel and has been certified as Halal by the Islamic Food and Nutrition Council of America (IFANCA).

5.1.3 EXTRACTION AND PURIFICATION OF DHASCO. The DHASCO oil is extracted from the algal biomass and processed using methods and procedures that have been well established in the edible oils industry. In order to protect this DHA-rich oil, which is much more prone to oxidation than typical vegetable oils, the Martek process has been designed to use the lowest effective temperatures and shortest times for each process step, and the oil is continuously protected from oxygen by nitrogen blanketing or vacuum.

The diagram in Figure 5.1.3-1 pictorially describes the DHASCO oil processing procedure. The oil is first extracted by blending the dried biomass with hexane in a

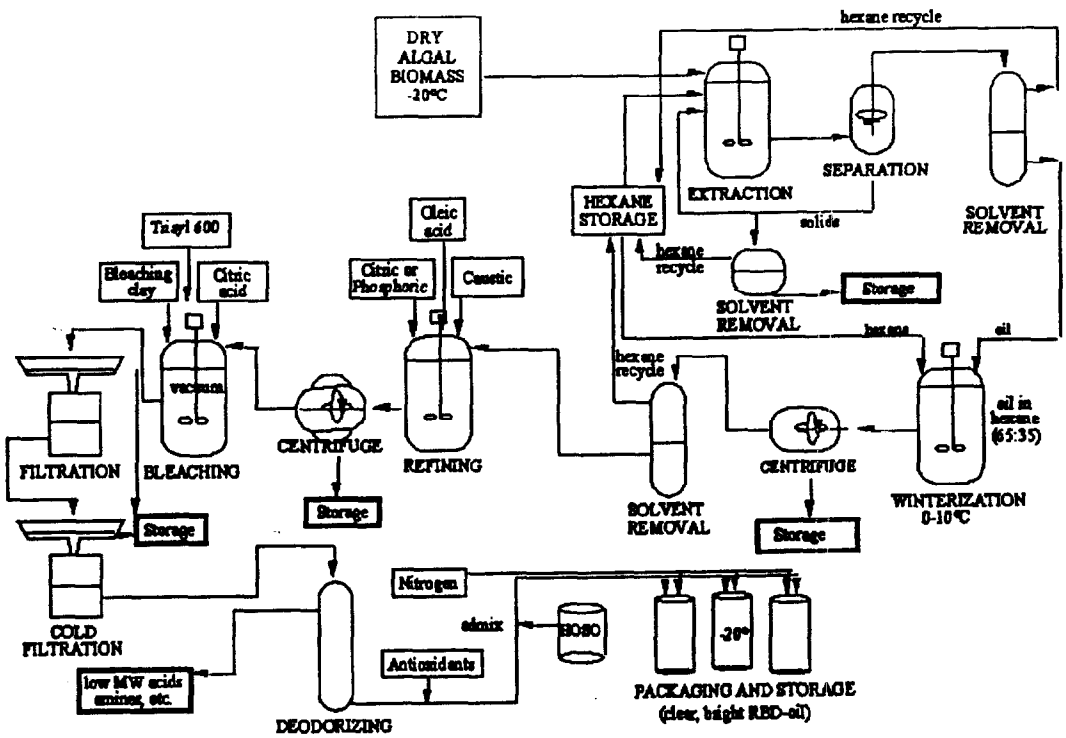

Figure 5.1.3-1. Flow chart of DHASCO oil processing.

continuous extraction process. The miscella (hexane:oil mixture) is separated from the de-oiled solids, filtered, and desolventized under vacuum to begin removal of the hexane. The oil is then winterized to remove a higher melting oil fraction by placing the miscella in a jacketed vessel, cooled and gently mixed. The chilled miscella is then centrifuged to remove higher melting solids and desolventized again to remove remaining volatiles. This winterized DHASCO is then refined to remove free fatty acids and phospholipids by mixing with citric or phosphoric acid while heating to facilitate removal of phospholipids. The free fatty acid level of the oil is adjusted using oleic acid, and the acids are neutralized by addition of aqueous sodium hydroxide. The mixture is heated and then centrifuged to remove the phospholipids and soaps of free fatty acids from the refined oil. The refined DHASCO is transferred to a vacuum bleaching vessel where citric acid, Trisyl 600 (activated silica) and bleaching clay are added to adsorb any remaining polar materials

and pro-oxidant metals, and to break down lipid oxidation products. The mixture is heated under vacuum and filtered using filter aid. The refined bleached DHASCO is then clarified once again by chilling the oil prior to a filtration step to remove any solids. Finally, the oil is deodorized under vacuum using a thin-film, packed-tower, continuous deodorizer. The deodorized DHASCO is then diluted to a standard 40% docosahexaenoic acid concentration by the addition of high oleic sunflower oil and mixed with antioxidants (mixed natural tocopherols and ascorbyl palmitate). The oil is packaged in either nitrogen-purged containers and stored frozen or vacuum packaged. Following analytical release testing, a Certificate of Analysis is generated and included with each shipment.

5.1.4 PROCESS CONTROLS. All processes were set up using a Hazard Analysis Critical Control Point (HACCP) approach. They are documented according to current Good Manufacturing Practices (cGMP) and the identified critical control points (CCPs) are monitored. Laboratory personnel use laboratory notebooks to record the results of laboratory tests as well as sterility checks, whereas production personnel record the continuous batch monitoring results in the batch records themselves, according to cGMP.

Significant process related data points are recorded and plotted on control charts. These data points are regularly monitored and reviewed to ensure that they are within Martek's recognized standard processing parameters.

Batch records are maintained and reviewed on a regular basis by Quality Control personnel to ensure that they are complete and accurate representations of the production process. Quality Assurance personnel monitor the production records to ensure that batch process changes have been properly authorized, documented, and recorded in the records for each batch.

The Food and Drug Administration (FDA) has inspected the manufacturing plant in Winchester, Kentucky, on the following occasions by the following inspectors:

Inspection Dates	*Inspector*	*Citations*
October 13, 1998	Charles R. Jody	None
August 4, 1997	Charles R. Jody	None
August 1, 1996	Robert W. Hudson	None
July 15-17, 1996	Robert W. Hudson	None
April 3, 1996	Charles R. Jody	None
April 4, 1995	Charles R. Jody	None

Working seed from the master seed culture is shipped to the manufacturing plant in Winchester, Kentucky in one-ml cryopreservation vials, on an as needed basis, from the Martek laboratories in Columbia, MD. Each shipment of frozen seed is accompanied by a Certificate of Analysis, which provides product identification and other analytical information from laboratory notebook records maintained in Maryland. At the manufacturing plant in Kentucky, frozen seed is maintained in liquid nitrogen until thawed for use. The thawing of a seed culture vial and the initiation of the seed train is documented in laboratory notebooks maintained at the Kentucky plant.

In-process monitoring of the product for bacteriological contamination is done on a continuous basis throughout the fermentation process. Sterility checks are performed daily by plating sterile samples of the fermented broth. The plates are cultured and held for 96 hours. During this time the plates are checked daily for signs of contamination. Laboratory personnel and production operators also routinely visually inspect samples from the fermentor under the microscope.

Before harvesting the fermentor, the cultures are microbially evaluated for gram negative organisms. If gram negative contamination is confirmed on a plate, the batch is terminated and discarded, and the contaminating microorganism is identified internally through visual inspection or by tests, or the plates are sent to a private, certified laboratory for identification of the contaminant. This process allows identification of the likely source of the contaminant (air, water, etc.) so that the source can be eliminated. Cultures are also rejected if other organisms, identified in the plate assays, have significantly altered established growth parameters or overgrown the algal culture. Each harvested batch is isolated until the plates made from the harvest time samples have been held for 96 hours and examined.

Temperature, pressure, and flow rates are monitored and controlled within tight specifications during the oil extraction and processing stages. The oil is maintained under nitrogen atmosphere or under vacuum to protect it from oxidation. The majority of the processing is done at very low moisture levels which prohibits microbial growth. In those steps where water is introduced, softened water is used, and the water is removed in the next process steps by centrifugation and vacuum. All processes are undertaken under food grade cGMP compliance as designated by the US FDA. All reagents used during manufacturing are of acceptable grade for food use and are Kosher, as verified by the manufacturer's Certificate of Analysis. Samples of the oil are collected at each step of the processing for analysis of key control parameters such as peroxide value, free fatty acid and phosphorous concentrations, and fatty acid composition.

The final product is analyzed by a variety of standard oil quality assays to ensure purity and quality, and that it meets the specification range value for each test prior to release. The stability of the oils is measured by determining the peroxide value (PV), the primary measurement of oxidation in oils.

5.2 ARASCO

5.2.1 NATURAL HISTORY OF *M. ALPINA*.

ARASCO, the ARA enriched single cell oil, is synthesized by a common soil fungus, *Mortierella alpina*. Fungi are eukaryotic, nonphotosynthetic organisms that have a vegetative structure known as a mycelium (a multinucleate mass of cytoplasm enclosed within a rigid, multi-branched system of tubes). A mycelium normally arises by the germination and outgrowth of a single reproductive cell, or spore. Upon germination, the fungal spore puts out a long thread, or hypha, which branches repeatedly as it elongates to form the mycelium. Fungal growth is characteristically confined to the tips of the hyphae. The size of a single mycelium is not fixed; as long as nutrients are available, outward growth by hyphal extension can continue. Usually, asexual reproduction occurs by the formation of spores,

which are pinched off at the tips of the hyphae. The fungi comprise three major groups: the Phycomycetes, the Ascomycetes and the Basidiomycetes (224).

All Phycomycetes share two properties that readily distinguish them from the remaining classes of fungi. Firstly, their asexual spores are always endogenous, and secondly, their mycelium shows no cross walls except in regions where a specialized cell is formed from a hyphal tip (nonseptate mycelium). The Phycomycetes include a group known as the terrestrial Phycomycetes, which are inhabitants of soil. *Mortierella alpina* is one of the most common species of the soil inhabiting phycomycetes. It can be easily isolated from the soil and has been isolated from soils all over the world (224). Like all fungi, *Mortierella alpina* may be found associated with common root crops and may, therefore, be directly in the food chain of many mammals.

Mortierella species have been well studied for many years in isolated laboratory culture. The morphology, biochemistry and physiology are well documented in more than 25 references in the last 20 years. *Mortierella alpina* has been described in Japanese publications and patents as a potential source of arachidonic acid and as a consequence, it has been more recently the subject of many intensive laboratory investigations (11, 158, 216, 230). In none of these recent studies, nor in any earlier studies, has there been any report of significant pathogenicity or toxigenicity to humans or animals (79, 211) by *M. alpina.*

Only a few of the thousands of known species of fungi cause human diseases. In a clinical setting involving an already diseased or immunocompromized host, any fungus that grows at 37°C could be considered an opportunistic pathogen (65, 220). In 1966, *Mortierella alpina* was reported to be isolated from liver lesions of a calf in New Zealand (220) as a secondary infection. However, it has been questioned whether this fungus was indeed *Mortierella alpina,* or some other species of *Mortierella.* (211).

Although some fungal species have been reported to produce mycotoxins, the mycotoxin-producing fungi belong to the class of Deuteromycota, which differ clearly from the Zygomycetous fungi to which *Mortierella* belongs (138). There has never been any report of mycotoxin production from *M. alpina* or any other of the many species of the genus *Mortierella* .

5.2.2 ARASCO FERMENTATION PROCESS. The diagram in figure 5.2.2-1 pictorially describes the fermentation process for the production of ARASCO-containing biomass of *Mortierella alpina.* The *M. alpina* strain used for production of ARASCO originates from ATCC (ATCC # 32222) and has been selected for rapid growth and high levels of production of the specific oil. The overall process has recently been described in the literature (153). Master seed banks of the production strain are maintained under liquid nitrogen conditions at Martek, Gist-brocades and at the ATCC. A working seed stock prepared from this master seed bank is also maintained cryogenically. Upon initiation of a production run, an individual ampoule from a working seed is used to inoculate a shake flask. The medium used to grow *M. alpina* from the shake flask to production scale contains dextrose (Corn Products), potassium phosphate, (laboratory grade) and yeast extract or hydrolyzed vegetable protein (Universal Foods). The cultures are transferred to successively larger vessels based on a rise in pH to a specified point. Temperature, pH, air flow, pressure, agitation, dextrose concentration, and dissolved

oxygen are all monitored and controlled. The culture is harvested based on measurements of pH, dextrose concentration, fat content of the biomass, and arachidonic acid content of the fat. The culture is harvested by centrifugation, and the biomass is then vacuum dried. The dried biomass is flushed with nitrogen and stored in poly-lined boxes prior to being shipped to the oil processing facility or frozen storage facility.

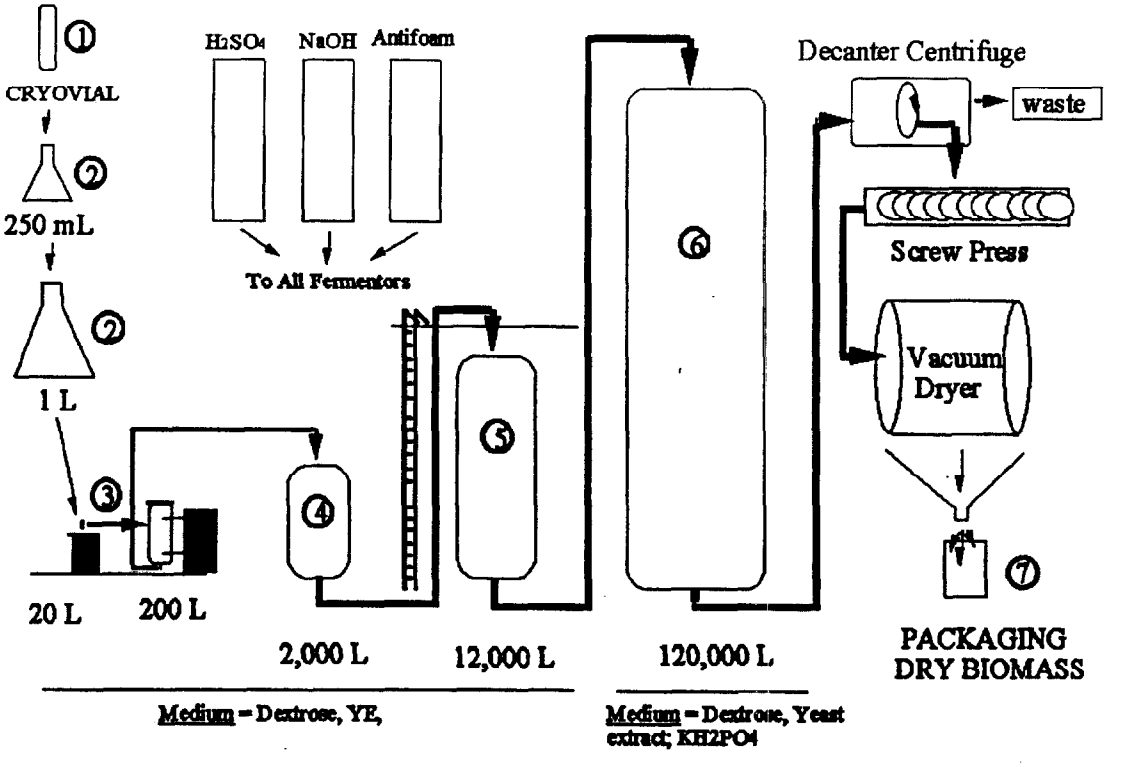

Figure 5.2.2-1. Flow chart of the stages of ARASCO fermentation scale-up and vacuum drying of the biomass.

All ingredients used in production are of food grade quality or better. This is verified from the manufacturers Certificate of Analysis and by Martek's policy of procuring ingredients only from verified reputable vendors of food grade quality products. In order to maintain kosher certification, Martek must also ensure that ingredients are only procured from recognized suppliers of products meeting strict kosher guidelines. The Orthodox Union must approve any deviations from this strictly controlled procurement source list prior to the introduction of an ingredient into the process from another supplier.

5.2.3 EXTRACTION AND PURIFICATION OF ARASCO. The ARASCO oil is extracted from the fungal biomass and processed using methods and procedures that have been well established in the edible oils industry. In order to protect this ARA-rich oil, which is much more prone to oxidation than typical vegetable oils, the Martek process has been designed to use the lowest effective temperatures and shortest

times for each process step, and the oil is continuously protected from oxygen by nitrogen blanketing or vacuum.

The diagram in Figure 5.2.3-1 pictorially describes the ARASCO oil processing procedure. The oil is first extracted by blending the dried biomass with hexane in a continuous extraction process. The miscella (hexane:oil mixture) is separated from the de-oiled solids, filtered, and desolventized under vacuum to reduce the volatiles. The crude ARASCO is then refined to remove free fatty acids, phospholipids and other impurities. The oil is first mixed with phosphoric acid, with heating, and the free fatty acids are neutralized by addition of aqueous sodium hydroxide. The mixture is heated and held before centrifugation to remove phospholipids, soaps of free fatty acids or other impurities from the refined oil. The refined ARASCO is transferred to a vacuum bleaching vessel, where Trisyl 600 (activated silica) and bleaching clay are added to adsorb any remaining polar materials and pro-oxidant metals and to break down lipid oxidation products. The mixture is heated under vacuum and filtered using filter aid. Finally, the oil is deodorized under vacuum using a thin film packed tower continuous deodorizer. The deodorized ARASCO is then diluted to a standard 40% arachidonic acid concentration by the addition of high oleic sunflower oil and mixed with antioxidants (mixed natural tocopherols and ascorbyl palmitate). The oil is packaged in either nitrogen-purged containers and frozen or vacuum packaged. Following analytical release testing, a Certificate of Analysis is generated and included with each shipment.

5.2.4 PRODUCTION OF *M. ALPINA* BY GIST-BROCADES. In a partnership Agreement with Gist Brocades (GB), Martek is also supplied with crude ARASCO for further processing in its plant. The fermentation process employed by GB is fundamentally similar to that described in Section 5.2.2. The manufacturing process is performed in accordance with cGMP and Kosher requirements. In the GB process, ammonia and ammonium sulfate are used as nitrogen sources instead of yeast extract or hydrolyzed protein. At the end of the fermentation process, the broth is pasteurized to kill the production microorganism and to inactivate any enzymes which could degrade the final oil quality. The broth is then filtered in a membrane filter press, and the cake is washed with process water. The filter cake is squeezed to remove excess water and fed into a single screw expander type extruder. The resulting material is then dried with a vibrating continuous fluid bed dryer. The dried material is extracted with hexane and the crude ARASCO oil is produced upon removal of the residual solvent by evaporation. This crude oil is delivered to Martek's oil processing facility for final purification according to Section 5.2.3 above. Incoming crude oil has to meet certain quality specifications before being accepted by Martek.

5.2.5 PROCESS CONTROLS. All processes are documented according to cGMP with CCPs. Laboratory personnel use laboratory notebooks to record the results of lab tests and sterility checks, whereas production personnel record the continuous batch monitoring results in the batch records themselves, according to cGMP. Significant process-related data points are recorded and plotted on control charts. These data points are regularly monitored and reviewed to ensure that they are within Martek's recognized standard processing parameters.

Batch records are maintained and reviewed on a regular basis by Quality Control personnel to ensure that they are complete and accurate representations of the production process. Quality Assurance personnel monitor the production records to ensure that batch process changes have been properly authorized, documented, and recorded in the records for each batch.

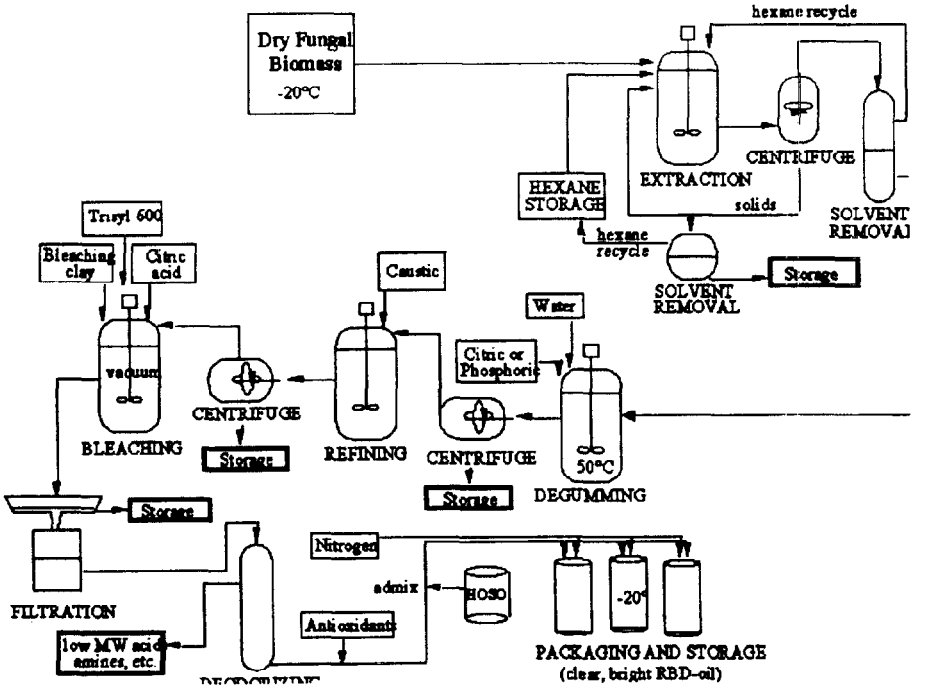

Figure 5.2.3-1. Flow chart of ARASCO oil processing.

The FDA has inspected the manufacturing plant in Winchester, Kentucky on the following occasions by the following inspectors:

Inspection Dates	*Inspector*	*Citations*
October 13, 1998	Charles R. Jody	None
August 4, 1997	Charles R. Jody	None
August 1, 1996	Robert W. Hudson	None
July 15-17, 1996	Robert W. Hudson	None
April 3, 1996	Charles R. Jody	None
April 4, 1995	Charles R. Jody	None

Working seed from the master seed culture is shipped to the manufacturing plant in Winchester, Kentucky in one-ml cryopreservation vials, on an as needed basis, from the Martek laboratories in Columbia, MD. Each shipment of frozen seed is accompanied by a

Certificate of Analysis, which provides product identification and other analytical information from laboratory notebook records maintained in Maryland. At the manufacturing plant in Kentucky, frozen seed is maintained in liquid nitrogen until thawed for use. The thawing of a seed culture vial and the initiation of the seed train is documented in laboratory notebooks maintained at the Kentucky plant.

In-process monitoring of the product for bacteriological contamination is done on a continuous basis throughout the fermentation process. Sterility checks are performed daily by plating sterile samples of the fermented broth. The plates are cultured and held for 96 hours. During this time the plates are checked daily for signs of contamination. Laboratory personnel and production operators also routinely visually inspect samples of the broth under the microscope.

Before harvesting the fermentor, the cultures are microbially evaluated for gram negative organisms. If gram negative contamination is confirmed on a plate, the batch is terminated and discarded, and the contaminating microorganism is identified internally through visual inspection or by tests, or the plates are sent to a private, certified laboratory for identification of the contaminant. This process allows identification of the likely source of the contaminant (air, water, etc.) so that the source can be eliminated. Cultures are also rejected if other organisms, identified in the plate assays, have significantly altered established growth parameters or overgrown the algal culture. Each harvested batch is isolated until the plates made from the harvest time samples have been held for 96 hours and examined.

Temperature, pressure, and flow rates are monitored and controlled within tight specifications throughout the oil extraction and processing stages. The oil is maintained under nitrogen atmosphere or under vacuum to protect it from oxidation. The majority of the processing is done at very low moisture levels which prohibits microbial growth; in those steps where water is introduced, softened water is used, and the water is removed in the next process steps by centrifugation and vacuum. All processes are undertaken under food grade cGMP compliance designated by the US FDA. All reagents used during manufacturing are of acceptable grade for food use and Kosher, as verified by the manufacturer's Certificate of Analysis. Samples of the oil are collected at each step of the processing for analysis of key control parameters such as peroxide value, free fatty acid and phosphorous concentrations, and fatty acid composition.

The final product is analyzed by a variety of standard oil quality assays to ensure purity and quality, and that it meets the specification range value for each test prior to release. The stability of the oils is measured by determining the peroxide value (PV), the primary measurement of oxidation in oils.

6. RELEASE SPECIFICATIONS AND BATCH ANALYSIS.

The release specifications for ARASCO and DHASCO and the Certificates of Analysis for these oils are shown in Figures 6-1 and 6-2. Current production lots of

ARASCO and DHASCO are standardized to approximately 40% ARA and DHA, respectively, prior to packaging. This is accomplished by diluting the final deodorized product with high oleic sunflower oil (HOSO). The HOSO is >80% oleic acid (18:1), so depending on the amount of dilution required, the final percentage of 18:1 in the oil can vary within a fairly wide range.

Due to the final production process used for the ARASCO and DHASCO oils (a high vacuum deodorization step at over 200 degrees C) the volatile solvents used in processing are no longer present in the final product at significant levels. However, for verification purposes, several lots of oil produced at Martek's Kentucky plant were tested for residual hexane used in both the DHASCO and ARASCO processes. DHASCO lots produced between May and September 1997 and the ARASCO lots produced in March of 1997 were used in the analyses. Samples were stored frozen in polyethylene bottles prior to analysis. None of the samples showed detectable amounts of this solvent (detection limit < 0.3 ppm).

CERTIFICATE OF ANALYSIS
DHASCO®
Lot Number 8800000220

Physical Description		Conforms
Appearance:	Clear, free flowing liquid at 40 C	yes
Color:	Yellow-dark orange	yes
Antioxidants:	0.025% ascorbyl palmitate, 0.025% tocopherols	yes

Chemical Analyses	Units	Minimum	Maximum	Results
Docosahexaenoic Acid	%	40	45	42.85
Docosahexaenoic Acid	mg/g	380	420	407.5
Eicosapentaenoic Acid	%		0.1	< 0.1
Free Fatty Acids	%		0.4	0.15
Peroxide Value	meq/kg		5	< 0.1
Moisture and Volatiles	%		0.1	0.02
Nonsaponifiables	%		3.5	1.93
Insoluble Impurities	%		0.1	< 0.01
Trans Fatty Acids	%		3.5	< 1.0

Elemental Analyses			
Arsenic	ppm	0.5	< 0.5
Cadmium	ppm	0.2	< 0.1
Chromium	ppm	0.2	< 0.1
Copper	ppm	0.1	< 0.02
Iron	ppm	0.5	0.02
Lead	ppm	0.2	< 0.1
Manganese	ppm	0.04	< 0.01
Mercury	ppm	0.2	< 0.04
Molybdenum	ppm	0.2	< 0.05
Nickel	ppm	0.2	< 0.1
Phosphorous	ppm	10	< 1
Silicon	ppm	500	41
Sulfur	ppm	100	25

Analysis Completed By: Central Analytical Laboratories, Inc.
Analytical Release By: _____
Product Release By: _____
Date: 6/21/99

rev. 6/21/99

Figure 6.1. Certificate of Analysis for release of DHASCO.

CERTIFICATE OF ANALYSIS
ARASCO®
Lot Number 8700000242

Physical Description		Conforms
Appearance:	Clear, free flowing liquid at 40 C	yes
Color:	Light-medium yellow	yes
Antioxidants:	0.025% ascorbyl palmitate, 0.025% tocopherols	yes

Chemical Analyses	Units	Minimum	Maximum	Results
Arachidonic Acid	%	38	44	42.96
Arachidonic Acid	mg/g	380	420	409
Eicosapentaenoic Acid	%		0.4	< 0.1
Free Fatty Acids	%		0.4	0.11
Peroxide Value	meq/kg		5	0.41
Moisture and Volatiles	%		0.1	0.03
Nonsaponifiables	%		3.5	1.36
Insoluble Impurities	%		0.1	< 0.01
Trans Fatty Acids	%		3.5	< 1
Elemental Analyses				
Arsenic	ppm		0.5	< 0.5
Cadmium	ppm		0.2	< 0.1
Chromium	ppm		0.2	< 0.1
Copper	ppm		0.1	< 0.02
Iron	ppm		0.5	0.04
Lead	ppm		0.2	< 0.1
Manganese	ppm		0.04	< 0.01
Mercury	ppm		0.2	< 0.04
Molybdenum	ppm		0.2	< 0.05
Nickel	ppm		0.2	< 0.1
Phosphorous	ppm		10	< 1
Silicon	ppm		500	280
Sulfur	ppm		100	3

Analysis Completed By: Central Analytical Laboratory
Analytical Release By: _____
Product Release By: _____
Date: 9/15/98

rev. 10/21/98

Figure 6.2. Certificate of Analysis for release of ARASCO.

7. SAFETY STUDIES

7.1 ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION (ADME). DHASCO and ARASCO are triglyceride oils, and the ADME of triglyceride oils is well known. Regardless of the source of fat or oil, triglycerides represent the principal source of dietary lipid for humans. A general review of the ADME of fats and oils can be found in standard biochemistry textbooks (168, 184). The main points are presented below:

Absorption. Ingested triglycerides are generally emulsified in the stomach and upper intestine (duodenum) where they are mixed with bile salts and enzymatically hydrolyzed by pancreatic lipase. The pancreatic lipase has a high degree of specificity for the Sn-1 and Sn-3 positions of the triglyceride, and the digested triglycerides are generally absorbed by the intestinal mucosa either as free fatty acids or as the Sn-2 monoglyceride.

Distribution. The intestinal mucosal cells absorb the free fatty acids and Sn-2 monoglycerides and "retailor" these components into new triglycerides and phospholipids. Triglycerides accumulate in large 1 mm droplets containing about 88% triglyceride, 8% phospholipid, 3% cholesterol ester, and 1-2% protein (apolipoprotein B-48) which are ejected from the gut mucosal cells by exocytosis as chylomicrons (CMs). These CMs are too large to cross the basement membranes of capillaries, so they enter the large-pored lacteal (lymph) vessels and leave the intestine with lymph via the thoracic duct before being dumped into venous circulation. The CMs are then tagged with ApoE and Apo C following an interaction with circulating HDL. This makes the CMs vulnerable to attack by vascular lipoprotein lipase, which hydrolyses the triglyceride, releasing individual fatty acids to the tissues for metabolism. CM remnants are resorbed by the liver, via the ApoE receptor. Excess fat and ApoB-100 (+ApoE and ApoC) results in VLDL synthesis in liver. VLDL is also attacked by lipoprotein lipase, providing free fatty acids to the tissues.

Metabolism. Fatty acids are building blocks for new membranes as well as energy rich fuel sources for cellular metabolism. In adipose cells, for example, the free fatty acids, produced by lipoprotein lipase, are retailored to triglycerides and stored as fat (triglyceride) for later use. Most of the fatty acids in tissues throughout the body, however, are oxidized by the well-known ß-oxidation pathway to produce 2-carbon substrates for the Kreb's cycle. The Kreb's metabolic cycle produces CO_2 and reducing power in the form of NADPH. NADPH is then oxidized in the mitochondria to produce ATP, releasing two electrons (added to O_2 to make H_2O). The ATP produced in this way provides energy for all cellular functions.

Excretion. The lipids consumed during a meal are virtually all metabolized and excreted as carbon dioxide and water. In cases of malabsorption due to certain pathologies (*eg.*, pancreatic insufficiency, short bowel, etc.), the triglycerides may not even enter the body and are excreted in the stools.

ADME of DHASCO and ARASCO specifically. DHA is absorbed in a manner similar to triglycerides found in other dietary fats and oils. A number of human studies have demonstrated the bioavailability of the DHA and ARA from DHASCO and ARASCO in normal adults (63, 131, 165, 186, 239) as well as in patients with various

metabolic disorders (69, 98, 122). Bioavailability is generally observed by the elevation of levels of DHA and ARA in red blood cells and/or serum phospholipids after dietary intake of the triglycerides DHASCO and/or ARASCO. Makrides *et. al.* (165) have also demonstrated bioavailability of DHA from dietary DHASCO in lactating women by the elevation of DHA in their breast milk lipids in a linear, dose dependent fashion with respect to the intake level. Both DHA and ARA are found circulating in the blood either as constituent parts of the erythrocyte membranes (*i.e.*, as membrane phospholipids), or in a non-cellular form as either triglyceride or serum phospholipids. In a recent study with human infants and using the metabolic balance method, Carnielli *et. al.* (51) demonstrated that about 80% of the DHA and ARA is absorbed when provided in a formula in the form of DHASCO and ARASCO. This value was identical to the absorption rates of ARA and DHA from the triglyceride in human milk. Starting with a 13C-labeled DHASCO, Sauerwald *et. al.* (209) have also shown that DHA is taken up from the gut, transported through the vasculature, and ultimately appears in the breast milk of lactating women at rates that were not different from any other fatty acids. These data are similar to those reported by Croset and coworkers (66) who also used 13C-labeled DHASCO to study the metabolic fate of the DHA in both rats and humans.

DHA and ARA are distributed throughout the body, but are found in higher concentrations in specific tissues such as brain, eye, testes, and heart. Studies in both rats (27, 150) and mice (245) have indicated that the former three tissues are resistant to changes in fatty acid composition in mature animals, even when provided with very large doses of DHA or ARA. Other tissues such as liver, red muscle, cardiac muscle, and adipose can vary in DHA and ARA content over a wide range, depending on the circulating levels of these fatty acids. This characteristic, however, is not unique to DHA and ARA, but is similar for other fatty acids as well.

7.2 METABOLISM AND BIOTRANSFORMATION. The metabolism of DHA and ARA is somewhat different from other dietary fats. Whereas most 16- and 18-carbon dietary fats are catabolized completely to CO2, H2O and energy, the catabolic rate of ARA, and particularly DHA, is very slow under most circumstances. Furthermore, free ARA can act as a precursor for lipoxygenase or cyclooxygenase reactions producing various omega-6 eicosanoids (*eg.*, thromboxane A2, prostaglandin E2, etc). One might expect that the very slow catabolic rate of DHA would lead to its accumulation in the body. Such an accumulation is reflected in the elevation of tissue contents of DHA when the body mass is not changing (*i.e.*, in adults). In a growing infant, on the other hand, the body mass is also increasing at a rapid rate, so even the maintenance of tissue DHA and ARA levels requires constant input of these components from the diet. The problem of declining tissue DHA and ARA levels in infants fed formulas without supplemental DHA and ARA is well known and was previously discussed in Section 2.

The biosynthesis of long chain polyunsaturated fatty acids (LC-PUFAs -- greater than 18 carbons in length) involves both the sequential addition of two-carbon units to an 18-carbon precursor, and the activity of Δ5- and Δ6-desaturases (Figure 7.2). The desaturation steps are thought to be rate limiting in this process. The 18-carbon, essential fatty acids precursors to ARA and DHA are linoleic acid (LA) and alpha linolenic acid

(ALA), respectively. The last steps in the biosynthesis of DHA have recently been demonstrated to be quite unique. A 24-carbon, hexaene precursor (C24:6(Δ6, 9, 12, 15, 18, 21)) is transported into the peroxisome and, through one step of beta oxidation, the 22-carbon DHA (C22:6(Δ4, 7, 10, 13, 16, 19)) is formed (244).

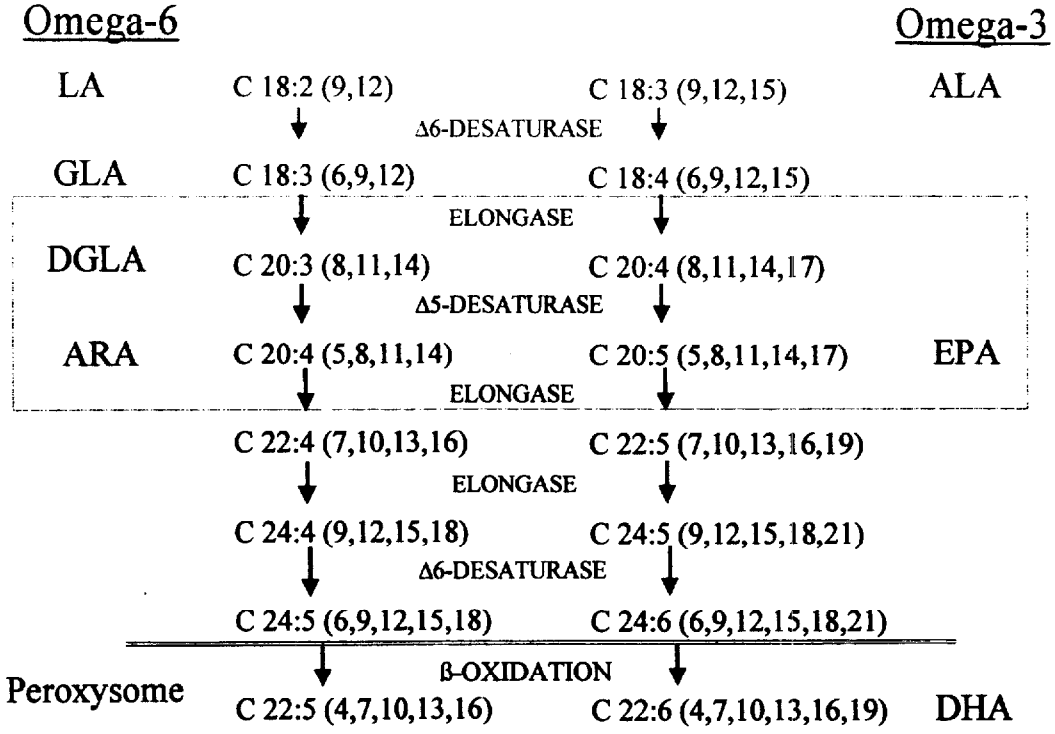

Figure 7.2. The omega-6 and omega-3 fatty acid biosynthetic pathways.

Different mammalian species have different abilities to form DHA from the essential fatty acid precursor ALA (204). Mammals that rely strictly on plant material for sustenance (*eg, Muscus*) have no access to preformed DHA in the diet, and are generally quite effecient at the conversion of ALA to DHA. On the other hand, some animals that are obligate carnivores (*eg.*, felines) have no ability to convert ALA to DHA and rely completely on preformed DHA in the diet for brain development (191). Humans have some ability to convert ALA to DHA, but only at a slow rate. This makes it difficult to find an accurate model for assessing the neurological consequences of the presence or absence of preformed DHA in the diet. Most clinical investigators have, therefore, analyzed the consequences of total omega-3, or omega-6, deficiencies for extrapolating to the human condition.

Few measurements have been made in man to assess abilities to elongate and desaturate ALA to DHA, or LA to ARA *in vivo*. Studies looking at the effect of dietary limitations (or excesses) of omega-6 and/or omega-3 fatty acids can provide a partial answer. Only through the use of stable isotopically-labeled precursor fatty acids, however, can accurate measurements of elongation and desaturation be made. Unfortunately, there have been very few of the latter studies (33, 52, 108, 146, 205, 210). With particular reference to infants, however, the data seem clear and consistent. Neither

preterm nor full term infants have the ability to elongate and desaturate dietary precursors to the extent needed to match the tissue accretion rates of babies receiving preformed DHA and ARA delivered in their mother's milk. This conclusion comes from three basic observations:

1) in every study (36 reported) where the blood lipids of a breast-fed baby is compared to a standard formula fed baby, the circulating DHA and ARA levels are significantly lower (typically 50% lower) in the formula-fed infants in spite of excesses of the precursors LA and ALA in the formula (see Table 2.1-1);
2) studies looking at DHA accretion in the brain tissues of infants *post mortum* have demonstrated that the DHA contents of the brains of standard formula-fed infants was much lower than those of breast-fed infants (97, 136); and
3) using stable isotope precursors, researchers demonstrated that the fractional synthetic rate for DHA in a human infant is far too slow to account for the required accumulation of DHA in the brain (205). In this case, the required accumulation is established by the gold standard of breast-fed babies.

The above three observations clearly indicate that LA and ALA are not adequate precursors for the ARA and DHA demands of a newborn infant. Furthermore, unless preformed ARA and DHA are added to an infant formula, one cannot expect to achieve normal rates of DHA and ARA accretion in the tissues, especially the brain and eyes, of that infant. Although we cannot definitively say that a slow rate of DHA accretion in the brain of an infant will have long term subtle neurological consequences, several recent well-controlled studies support this possibility. Consequently, it seems prudent to provide a nutritional source that matches the DHA and ARA accretion rates of the normal breast-fed infant rather than risk any unknown consequences of significantly slowing this accretion rate.

7.3 RETROCONVERSION OF DHA INTO EPA. Retroconversion is an enzymatic process whereby long-chain fatty acids are converted to their related shorter-chain fatty acids through the incremental removal of two-carbon units from the molecule. This process is similar to beta-oxidation, but in this case, the reaction stops after the removal of two or four carbons. The two-carbon units are removed from the carboxyl end of the fatty acid leaving the methyl end (omega) intact, so fatty acids from the same family are synthesized by this process. Thus, 22:4n-6 is thought to be retroconverted into ARA (20:4n-6) and erusic acid (22:1n-9) into 20:1n-9.

The retroconversion of DHA into EPA, however, is more complicated. It involves the removal of a double bond at the Δ4 position in addition to the removal of a two-carbon unit (i.e., C22:6 is converted to C20:5). Consequently, there is some controversy as to whether the elevation of EPA levels seen during supplementation of DHA is really due to the retroconversion of preformed DHA into EPA, or simply due a feedback inhibition of DHA biosynthesis at a point beyond EPA, thereby resulting in a build up of EPA as an intermediate in the pathway.

Whether or not the biochemical mechanism involves retroconversion or feedback inhibition, clinical data have established that large oral doses of DHA consumed by humans and animals lead to an increase in circulating EPA levels (8, 27, 63, 64, 186, 239).

In experiments feeding [13C]-labeled DHA, Brossard et al. (34) reported that labeled EPA and DPAn-3 were synthesized starting from labeled DHA. Although this seems to support the hypothesis of retroconversion, these authors calculated from their data that the conversion rate to EPA in humans receiving normal dietary levels of DHA was vanishingly small (only about 1.4%). In contrast, they also reported that rodents in the same experiment exhibited a much higher intrinsic rate of DHA retroconversion to EPA (maximum of 9%). The human clinical data available to date further indicates that the apparent retroconversion rate of DHA into EPA appears to be dramatically dependent on the amount of DHA in the diet. Holub and colleagues, for example, calculated that at very high chronic oral doses in humans (>1.2 g DHA/day), the apparent retroconversion rate could be as high as 12% (63, 64).

High levels of EPA may perturb the ARA balance in infants, and in one early clinical study using a high EPA fish oil supplementation, the high EPA levels resulted in slower growth in preterm infants (47). Thus, the possibility of retroconversion of DHA into EPA could be of concern. For this reason, only DHA sources with minimal levels of EPA (preferably no EPA) have been recommended for the supplementation of infants formulas. Once again, however, we should look to human milk as the gold standard. DHA is normally provided to a growing infant in the form of human milk so if retroconversion was taking place in infants, it is the natural process. Nevertheless, several clinical studies with preterm and full term infants have been completed and provide us with the empirical answer. The very low levels of DHA used in supplemented infant formulas (< 1.0 % of fatty acids) or in mother's milk, do not result in any significant increases in EPA in the plasma or red blood cells when the supplement used in the formulas was a mixture of DHASCO and ARASCO (22, 53, 61, 91).

In conclusion, there is no concern over elevation of serum EPA levels when infants are fed formulas supplemented with both DHASCO and ARASCO at levels comparable with those in human milk.

7.4 NONSAPONIFIABLE FRACTION. The nonsaponifiable fractions of DHASCO and ARASCO represent only a small fraction of the overall mass of the oils (*i.e.*, typically no more than 1.5% of the total oil mass), and are a mixture of several minor components. Like most nonsaponifiable fractions from vegetable oils, these components are predominantly sterols. The sterols from the nonsaponifiable fractions of DHASCO and ARASCO have been fractionated and analyzed by two independent laboratories, and the results are provided in Tables 4.1-3 and 4.2-3. The values given in these tables are in general agreement with values reported in the literature. Some of these sterols, such as desmosterol, are already known to be in an infant's diet via its mother's milk (141). 4-Methyl sterols are normal intermediates in cholesterol biosynthesis, and humans have the enzymatic capability to convert 4-methyl sterols into 4-desmethyl sterols like cholesterol (93, 116). Microalgae such as *C. cohnii* are commonly found in the food web of filter feeders like mollusks (*eg.*, oysters and clams), crustaceans (*eg.*, shrimp and lobster), and certain fish, and therefore, these sterols can also be found in such animals. As a result, these sterols have been in the human food chain, both directly and indirectly, ever since man was eating marine foods.

Since there have been no literature reports on the effects of consumption of such 4-methyl sterols directly, a feeding study was undertaken using a high concentration of the nonsaponifiable fraction of *C. cohnii* in rats. This study compared the effects of consuming a high concentration of the nonsaponifiable fraction from DHASCO with the nonsaponifiable fraction of soybean oil, which contains predominantly sitosterol (151). Although sitosterol is known to inhibit the re-uptake of cholesterol from the gut, the nonsaponifiable fraction of DHASCO did not show a similar effect. Furthermore, when a very large portion (0.5% by weight) of the diet of rats was comprised of this nonsaponifiable fraction, there was no adverse effect on growth, general physiology, or the gross organ morphology in the recipient animals. The study concluded that there were "*no toxicological effects on the rats of consuming up to 0.5% of their diet in the DHASCO nonsaponifiables*". This dose would be equivalent to giving the rat a diet containing about 50% by weight pure DHASCO.

7.5 TOXICOLOGICAL STUDIES (NON-HUMAN). A large number of safety tests have been conducted on ARASCO and DHASCO oils by several different organizations. As a result, there is a large degree of redundancy in the standard toxicological assessments. This redundancy allows for an unprecedented and extremely valuable assessment of these oils. The studies include *in vitro* genotoxicity assays and a variety of animals studies, such as acute, subchronic, developmental and multigenerational reproductive studies. Since these components represent macronutrients, all studies included appropriate positive as well as negative controls. All studies reported herein were conducted in Good Laboratory Practice (GLP)-compliant laboratories following guidelines outlined by the FDA in *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Foods*, commonly referred to as Redbook I, or from their draft Redbook II. The audited summaries from each toxicology study appear in Appendix 4. Only the significant findings will be presented here. These data have also been reported in a number of publications and summarized by Kyle and Arterburn (154).

At the outset, it is important to recognize that DHASCO and ARASCO are macronutrients, not micronutrients, vitamins, or drugs. Safety testing of macronutrients poses several problems (26). For example, it is often difficult to distinguish whether an observed response is related to a toxicological effect of the test material, or due to a dietary deficiency of some other component caused by the use of such large amounts of the test macronutrient in the diet. Clearly, one cannot achieve safety margins of greater than 50-fold for a macronutrient that comprises more than 2% of the diet. Finally, when a macronutrient test material is added to the diet at a very high dose, one must carefully distinguish a truly toxicological response due to the test material from a normal physiological response resulting from the high dietary load of that particular macronutrient. For example, is the elevation of serum cholesterol in a human who changes their diet from one containing 25% of dietary calories as fat to one with 45% of dietary calories as fat a toxicological response to the particular fat used, or a normal physiological response to increasing total dietary fat? With this caveat, the toxicological studies completed on DHASCO and ARASCO are summarized below.

7.5.1. ACUTE TOXICITY STUDIES. Several studies have shown that at acute oral doses of 20 grams of DHASCO or ARASCO/kg body wt in rats, there were no deaths (Table 7.5.1). Soft stools were typically noted in the first day, but all animals gained weight during the two-week post-dosing period. Soft stools are a normal and expected consequence of a large, single dose of any fatty substance, and it was not considered to be an adverse event. Since no animals died, acute oral LD50 values could not be determined, but based on the lack of toxicity in the tests, they were greater than 20 g/kg body wt/day for each oil.

Table 7.5.1. Acute toxicity studies with DHASCO and ARASCO in Rats.

Test Material	*Sponsor Study Date*	*Dose (g kg bw)*	*Mortality (male female)*		*LD50*	*Reference*
DHASCO	Martek	20	0/5	0/5	>20 g/kg bw	(27)
DHASCO	Martek	20	0/5	0/5	>20 g/kg bw	(100)
C. cohnii algal biomass	Martek	7	0/5	0/5	>7 g/kg bw	(103)
C. cohnii biomass	Martek	7	0/5	0/5	>7 g/kg bw	(104)
Delipidated C. cohnii biomass	Martek	10	0/5	0/5	>10 g/kg bw	(101)
Delipidated algal biomass	Martek	6	0/5	0/5	>6 g/kg bw	(105)
ARASCO	Martek	20	0/5	0/5	>20 g/kg bw	(27)
ARASCO	Martek	20	0/5	0/5	>20 g/kg bw	(102)
ARASCO	Gist-brocades	18.2	0/5	0/5	>18.2 g/kg bw	(117)
Fungal biomass	Martek	5	0/5	0/5	>5 g/kg bw	(99)
Delipidated fungal biomass	Martek	4	0/5	0/5	>4 g/kg bw	(106)
Microencapsulated ARASCO/DHASCO blend	Martek	5	0/5	0/5	>5 g/kg bw	(107)

7.5.2 SUB-CHRONIC (28-63 DAY) TOXICITY STUDIES. In several independent studies, DHASCO or ARASCO was administered by gavage, or as a dietary admixture, daily for 28 or 63 days using doses of 0.025 to 9.4 grams of test material /kg body wt/day (Table 7.5.2). The No Adverse Effect Levels (NOAEL) were the highest doses tested for each individual oil during the specific study (up to 2.5 g oil/kg body wt/day for ARASCO and 1.25 g/kg bw/day for DHASCO) and for the combined oils (up to 9 g/kg body wt/day). At the highest dose levels there were some recurrent findings (see section 7.5.9), but these were not considered to be adverse effects of the test material because they were not accompanied by changes in histology or clinical chemistry. Rather, they were considered normal physiological responses to high doses of polyunsaturated fatty acids and to the use of artificial diets in some cases.

Table 7.5.2. Sub-chronic (28 to 63 day) oral safety studies with DHASCO and ARASCO in Rats.

Test Material	*Sponsor*	*Highest Dose (g/kg bw/day)*	*Details*	*Conclusions*	*Reference*
ARASCO/DHASCO 1.5:1 blend	Wyeth Ayerst	1.2	63 days in the diet; 10 rats/sex/dose	Not toxic	(88)
DHASCO	Martek	1.25	28 days by gavage; 5-10 rats/sex/dose	No toxic	(27)
ARASCO	Martek	2.5	28 days by gavage; 5-10 rats/sex/dose	Not toxic	(27)
ARASCO	Gist-brocades	3.0	28 days by gavage; 10 rats/sex/dose	Not toxic	(117)
ARASCO/DHASCO 2:1 blend	Martek	3.75	28 days by gavage; 5-10 rats/sex/dose	Not toxic	(27)
ARASCO/DHASCO 2:1 blend	Mead Johnson	9.1/9.4	28 days in the diet; 10 rats/sex/dose	Not toxic	(247)
Algal biomass	Martek	5	28 days in diet, 5-10 rats/sex/dose	Not toxic	(249)

7.5.3 SUB-CHRONIC (90-DAY) STUDIES. DHASCO and ARASCO were administered by gavage or as a dietary admixture for 90 days at doses up to 8.9 grams of test material/kg body wt/day with 20 rats per sex per dosing group. The No Adverse Effect Levels as determined following a critical evaluation of the data by the study site toxicologists, were the highest doses tested for each material in all studies (up to 2.5 grams of ARASCO and 1.25 grams DHASCO per kg bw per day and up to 8.9 g/kg body wt/day for combined oils) except for the Gist-brocades study (see Table 7.5.3) (8, 36, 150). A NOAEL of 8.9 gm/kg/day (about 6 grams ARASCO and 3 grams of DHASCO) represents at least a 60-fold excess of this macronutrient from the intended use level of ARASCO, and a 50-fold excess from the intended use level of DHASCO. At the highest dose levels, there were some findings (see section 7.5.9) that were not considered to be adverse test material because they were not accompanied by changes in histology or clinical chemistry. Rather, they were considered normal physiological responses to high doses of polyunsaturated fatty acids and to the use of artificial diets in some cases. Some of these studies also involved detailed assessments of neurotoxicity (8, 143) as outlined by the FDA Redbook II Guidelines, and other studies also involved an *in utero* supplementation phase (36).

7.5.4. CHRONIC TOXICITY/CARCINOGENICITY STUDIES. Based on *in vitro* toxicological information (see Section 7.5.6) and knowledge of the macronutrient nature of the test material, it was determined that chronic toxicity/carcinogenicity studies were not necessary for this nutrient.

Table 7.5.3. Sub-chronic 90-day toxicity studies with DHASCO and ARASCO in Rats.

Test Material	*Sponsor*	*Highest Dose (g/kg bw/d)*	*Details*	*Findings*	*Reference*
DHASCO	Martek	1.25	gavage, full neurotox assessment	Not toxic	(8)
DHASCO/ARASCO blend	Numico	2.0	in diet	Not toxic	(113)
ARASCO	Martek	2.5	gavage, full neurotox assessment	Not toxic	(150)
DHASCO/ARASCO 1.5:1 blend	Wyeth Ayerst	2.5	in diet	Not toxic	(88)
ARASCO	Gist-brocades	4.9	in diet, *in utero* phase, full neurotox assessment	Not toxic	(157)
DHASCO/ARASCO 2:1 blend	Mead Johnson	8.9	in diet, *in utero* phase, full neurotox assessment	Not toxic	(36)
Algal biomass	Martek	5.8	in diet, full neurotox assessment	Not toxic	(225)

7.5.5 REPRODUCTIVE/DEVELOPMENTAL TOXICOLOGY STUDIES. Developmental toxicology studies were conducted with DHASCO and ARASCO administered by gavage (Table 7.5.5). There were no adverse developmental effects at the doses tested as determined by the independent study site toxicologists.

Table 7.5.5. Developmental and reproductive toxicity studies with DHASCO and ARASCO in Rats.

Test Material	*Study Type*	*Sponsor*	*Highest Dose (g/kg bw/d)*	*Details*	*Findings*	*Reference*
DHASCO	Developmental	Martek	1.25	gavage 25/sex/dose	Not toxic	(7)
ARASCO	Developmental	Martek	2.5	gavage 25/sex/dose	Not toxic	(7)
Algal biomass	Developmental	Martek	4.3	in diet 20/sex/dose	Not toxic	(242)
Algal biomass	Reproductive	Martek	8	in diet 20/sex/dose	Not toxic	(241)

7.5.6 GENOTOXICITY STUDIES - *IN VITRO*. The test materials were determined to be neither mutagenic, clastogenic, nor genotoxic through a number of standard *in vitro* mutagenicity tests (Table 7.5.6) when tested with and without *in vitro* metabolic activation using rat liver microsomal S9 fractions.

Table 7.5.6. *In vitro* genotoxicity studies with DHASCO and ARASCO.

Test Material	*Test*	*Doses*	*Findings*	*Reference*
DHASCO	AMES	100-5,000 ug/plate	Not mutagenic	(28, 29)
DHASCO	Forward mutation	750-5,000 ug/ml	Not mutagenic	(28, 29)
DHASCO	Chromosomal aberration	1250-5,000 ug/ml	Not clastigenic	(28, 29)
ARASCO	AMES	100-5,000 ug/plate	Not mutagenic	(28, 29)
ARASCO	Forward mutation	750-5,000 ug/ml	Not mutagenic	(28, 29)
ARASCO	Chromosomal aberration	1250-5,000 ug/ml	Not clastogenic	(28, 29)
Algal biomass	AMES	33-5,000 ug/plate	Not mutagenic	(156)
Algal biomass	Forward Mutation	30-1,000 ug/ml	Not mutagenic	(57)
Algal biomass	Chromosomal Aberration	50-1,000 ug/ml	Not clastogenic	(177)

7.5.7 SPECIAL STUDIES. Tests for potential dinoflagellate toxins were conducted even though there is no indication from historical data that this species produces any toxins. DHASCO was given by intraperitoneal injection to mice at doses as high as 10% of the animal's body weight without toxicological activity. Analytical and biochemical tests for specific dinoflagellate-related toxins in the DHASCO oil were all negative. In addition, no endotoxins were identified in studies using the complete biomass as a test material when provided intraperitoneal injections of up to 1.25 g/kg[15].

7.5.8 STUDIES WITH WHOLE ALGAL BIOMASS. In addition to studies on the oil product produced using the production process outlined in Sections 5 and 6, a further level of safety testing was undertaken using the intact, unprocessed biomass directly as a food component. In such an instance, all available potential toxicants would be imposed on the animals. When fed in doses as large as 7 g/kg bw/d, the biomass showed no acute toxicity (Table 7.5.1). At a sub-chronic dose of 5 g/kg bw/day for 28 days, the biomass likewise showed no toxic indications (Table 7.5.2). More extensive developmental and multigenerational toxicology tests were also undertaken with the intact biomass (Table 7.5.5). At a dose of 8 g biomass/kg bw/day in the diets of rats, there were no dose-related toxicological findings in reproductive parameters, over three generations. Since these products are intended for use in the perinatal period, additional developmental toxicity testing was completed at doses of 4.3 g biomass/kg bw/day and there were again no test material related toxicological findings at that dose. Finally, the intact biomass also revealed no mutagentic or clastogenic effects using *in vitro* assays (Table 7.5.6). In conclusion, not only does the processed oil show no toxicological activities of concern, but the parent biomass itself is equally free of toxicological findings.

[15] Notice of a Claim of Exemption from Premarket Approval, submitted to the Office of Premarket Approval, Food and Drug Administration by Wyeth Ayerst Laboratories on August 27, 1998.

7.5.9 RECURRENT FINDINGS - RELATIVE ORGAN WEIGHTS. Out of thirteen[16] sub-chronic toxicity studies completed on these oils, five indicated a statistically significant increase in relative liver weights at the highest doses of ARASCO or ARASCO/DHASCO blends (Table 7.5.9-1). Because the findings were not consistent, nor was there any accompanying changes in liver histopathology or abnormally high levels of liver enzymes in the serum, the independent study pathologists separately, and unanimously, concluded that these were not adverse toxicological findings.

Because this phenomenon mght be considered an important toxicological finding, a detailed, simultaneous evaluation of the thirteen sub-chronic studies was undertaken to gain a better understanding of the relevance and consistency of this finding. A careful simultaneous evaluation of the liver-related clinical chemistries in all studies did not reveal any consistent dose-dependent effects. All trials contained both low fat and high fat controls, although the choice of control fat source varied (corn oil, soybean oil, canola oil, and high oleic sunflower oil). The high fat control was necessary to distinguish physiological responses to a high fat diet from specific test material related phenomena. (The total fat load in these studies were generally two to three times the normal level found in standard rat chow.) In addition, synthetic diets were used in some studies while others used standard chow, some mixed the oils directly into the diet, and others provided the oils by gavage at a specific dose based on animal weight. As a result, the trials represented a broad spectrum of designs, although the numbers of animals of each sex and multiple-dose approaches were generally consistent with FDA Redbook guidelines. Although some studies reported a statistically significant increase in the liver weights relative to body weights compared to high fat controls, none of the mean relative liver weights were outside the historical normal range (shaded bar in Figure 7.5.9-1).

Pathologists also noted a slight to moderate vacuolization (accumulation of lipid) in some, but not all, of the high dose treatment groups, but the incidence of this finding was not different than the high fat control group and was attributed to the high fat diet or the use of synthetic diets with high fat and carbohydrate, as has been previously reported in the literature (62, 120, 169, 214). No other histopathological changes (*i.e.*, necrosis , *etc.*) were observed consistently in any of the groups, and there were no consistent changes in clinical chemistry that would suggest toxicity. Three studies reported a decrease in albumin levels and/or total protein levels, but this finding was not consistent across studies, nor did the changes parallel increases in liver weights within or across studies.

[16] Excluding Wyeth 63-day and Numico 90-day studies for which detailed data was not available.

Table 7.5.9-1. The Effect of Dietary Supplementation With DHASCO and/or ARASCO at various doses (mg/kg/day) on the Relative Liver Weights (% of body weight) of Male and Female Rats.

Males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F*	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				2.87	2.87	3.22	3.32	3.35				3.53	3.53
H	3.55	3.55	3.55	2.9	2.9	3.23	3.1	3.23	4.08	4.08	4.08	3.33	3.33
25	3.54												
50		3.42											
100								3.27	3.98				
200												3.2	
300										4.11			
500	3.56				2.89								
600								3.21	4.37				
1000		3.59		3.03						4.08		3.35	
1150							3.3						
1250	3.61				2.98								
1500			3.88							4.3			
1600						3.39							
2000									4.36				
2300								3.37					
2500		3.89		3.12									
3000									4.25		4.28		
3750			4.11										
4050													
4500											4.47		
4900												3.27	
8550													
9100													3.36
9250						3.53							

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F*	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				2.89	2.89	3.53	3.25	3.64				3.15	3.15
H	3.56	3.56	3.56	2.89	2.89	3.5	3.03	3.31	3.74	3.74	3.74		
25	3.58												
50		3.63								3.9			
100								3.24	3.81				
200												2.87	
300										3.73			
500	3.56				2.86								
600								3.31	3.66				
1000		3.73		2.91						3.92		2.91	
1150							3.14						
1250	3.58				2.99								
1500			3.73							3.81			
1600						3.65							
2000									3.89				
2300								3.34					
2500		3.64											
3000									3.94		3.92		
3750			4.06										
4050													
4500											4.09		
4900													
8550													
9100													
9250													

Values in yellow represent the results of 13 separate toxicological studies; Highlighted values are statistically different from controls. Tox study labels indicate sponsor (MK, Martek; MJ, Mead Johnson; W, Wyeth/Ayerst; GB, Gist-brocades), duration (28 or 90 days), and test material (D, DHASCO; A, ARASCO; F, DHASCO+ARASCO; fo, fish oil). H, high fat control.

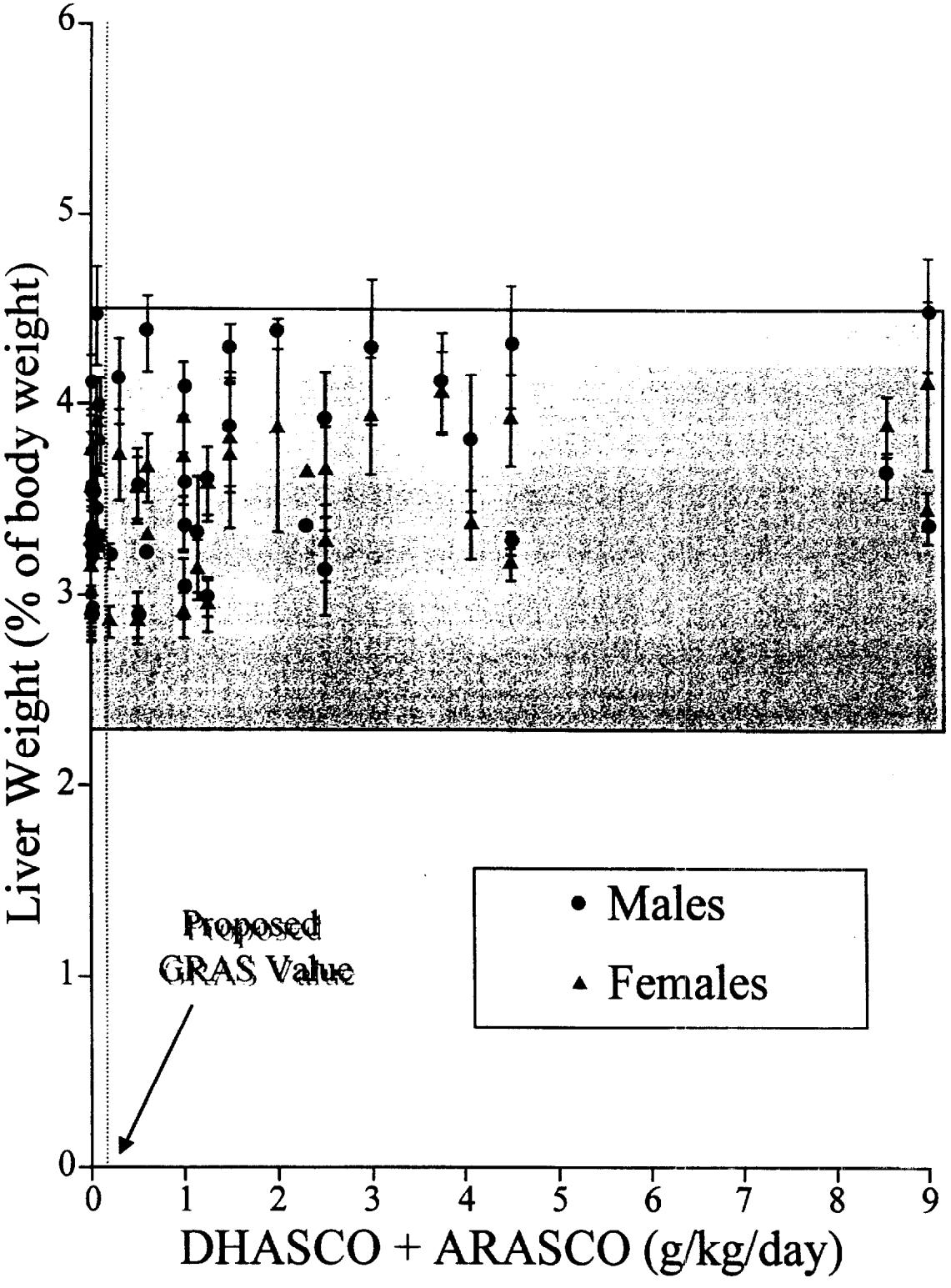

Figure 7.5.9-1. Changes in relative liver weights in rats with chronic consumption of DHASCO and ARASCO. Shaded area represents the historical control range.

A comprehensive survey of the published literature revealed that significant increases in relative liver weights in rats (75, 175, 176, 219, 223, 254, 256), mice](1, 70), Guinea pigs (179), and rabbits (199), following consumption of high doses of long chain polyunsaturated fatty acids (mostly from various fish oils, including Menhaden oil which has already been affirmed by the FDA as GRAS for many food uses) has been well established. The salient features of these studies are provided in Table 7.5.9-2. Regardless of the source of the PUFA, this table indicates that there is a consistent 20-40% increase in relative liver weights in response to the feeding of test fish oils at levels of 3-5% of the diet as LC-PUFA (sum of EPA + DHA). In this respect, DHASCO and ARASCO are no different. Table 7.5.9-3 presents a summary of the results of the toxicology studies preformed on DHASCO and ARASCO, in a manner that they can be compared directly to the literature values in Table 7.5.9-2. In most cases, the doses of DHASCO and ARASCO were lower than those for the fish oil, and there were no significant increases in relative liver weights at these low levels. Where doses equivalent to the fish oils in Table 7.5.9-2 were used (eg., Mead Johnson studies), responses similar to those reported for the fish oils were observed. Thus, we conclude that the increase in relative liver weights seen in some studies where very high levels of DHASCO or ARASCO were used, is consistent with a well established effect of the PUFAs themselves, and are not due to some unknown component unique to these oils. The fact that the same observation is noted for ARASCO, DHASCO, and various fish oils, all oils of completely different biological origin and composition (except for the PUFAs themselves), argues strongly that this is a PUFA effect independent of the source.

Table 7.5.9-2 Effect of Dietary Fish Oils on Liver Weights of Small Mammals

Species	*Study Reference*	*Test Oil*	*Control Oil*	*Duration (wks)*	*Dose (wt % of diet)*		*% Increase Liver Wt*	
					Test Oil	*LC-PUFA*	*Absolute*	*Relative*
Rats, female	(4)	Menhaden	Corn	8	17	3.5		
		Cod liver	Corn	8	19	3.2		
		Menhaden	Lard	5	17	3.5	18	
		Sardine	Lard	5	17	5.1	16	
		Cod liver	Lard	5	19	3.2		
		Menhaden	Lard	10	17	3.5	7	
		Sardine	Lard	10	17	5.1	16	
		Cod liver	Lard	10	19	3.2		
	(173)	Menhaden	Lard	5	17	3.5		
		Sardine	Lard	5	17	5.1		
		Cod liver	Lard	5	19	3.2		
		Menhaden	Lard	10	17	3.5	32	
		Sardine	Lard	10	17	5.1		
		Cod liver	Lard	10	19	3.2		
Rats, male	(256)	Menhaden	Safflower	10	20	3.4		nr
	(223)	Omega	Coconut/	3	1.4	0.1		5
		Pharma	Safflower/	3	2.8	0.2		8
		fish oil	tallow	3	5.6	0.3		
	(176)	Menhaden	Corn	10	24[17]	4.8	20	nr
	(255)	MaxEPA	Safflower	2	15	6	2	

[17] Estimated from information on energy percent of fat.

Species	Study Reference	Test Oil	Control Oil	Duration (wks)	Dose (wt % of diet) Test Oil	Dose (wt % of diet) LC-PUFA	% Increase Liver Wt Absolute	% Increase Liver Wt Relative
	(76)	EPA-EE	Palmitic		1.3[18]	1.2	13	4
		DHA-EE	Palmitic		1.3[2]	1.3	29	14
	(219)	Fish oil	Corn	4	17	4.4	18	17
Rats, male, lean	(175)	Menhaden	Coconut	10	18	3.6	13	13
			Safflower	10	18	3.6	18	
Rats, male, obese		Menhaden	Coconut	10	18	3.6	3	
			Safflower	10	18	3.6	-4	
Mice, female	(1)	DHA-EE	Safflower	8	1	1	nr	
Mice, male		DHA-EE		8	1	1	nr	
	(70)	Salmon	Low fat chow	0.4	10	5.3	nr	
				2			nr	
				3			nr	
Guinea pigs, male	(180)	Menhaden	Corn	4.5	10	2.0	nr	17
		Seal		4.5	10	1.3	nr	
		Shark liver		4.5	10	2.0	nr	
Rabbits, female	(199)	Menhaden	nr	129	14[20]	4.0		
				129	7[20]	2.0	11	
				129	0.7[20]	0.2	-8	-2

nr – not reported.
Shading indicates a significant difference between control and treated groups.

Table 7.5.9-3 Effect of DHASCO and ARASCO on Liver Weights of Rats

Study References	Test Oil(s)	Control Oil	Duration (wks)	Dose (wt % of diet) Test Oil	Dose (wt % of diet) LC-PUFA	% Increase in Liver Wt Absolute M	% Increase in Liver Wt Absolute F	% Increase in Liver Wt Relative M	% Increase in Liver Wt Relative F
(27)	DHASCO	HOSO	4	1.1[19]	0.4	4	1	2	0
	ARASCO	HOSO	4	2.3[22]	1.1	13	3	10	2
	Formulaid	HOSO	4	3.3[22]	1.5	18	14	16	14
(150)	ARASCO	HOSO	13	3.3[22]	1.8	3	10	9	
(8)	DHASCO	HOSO	13	1.7[22]	0.9	1	0	4	3
(247)	Formulaid	Canola	4	6.0	2.6	11	0		
				12	5.3	7	7	9	
(36)	Formulaid	Canola	13	6	2.8				
				12	5.6	17			
Wyeth study, unpublished	Formulaid	Soy	13	3.3	1.0	8	2	4	12
Gist-brocades study, unpublished	ARASCO	Corn	4	3.5	1.2	4	-2	4	5
	ARASCO + FO			5.7	1.8	7	2	9	10
Gist-brocades study, unpublished	ARASCO	Corn	13	7.5[22]	3.0	2	8	-2	9
	ARASCO + FO			13.0[22]	4.4	2		-2	

HOSO – high oleic sunflower oil; FO – fish oil.
Shading indicates a significant difference between control and treated groups.

[18] Estimated from food intake and average weights based on oils being gavaged at 1 g/kgbw/day.
[19] Oil administered by gavage. Doses were calculated using animal weights and food intakes.

Why do high doses of dietary LC-PUFAs, regardless of the source, lead to increases in relative liver weight? The literature offers several possible explanations. Polyunsaturated fatty acids are well known to down-regulate lipogenesis (fat biosynthesis), thereby slightly decreasing the total body weight without affecting lean body mass. This is often difficult to detect in a growing animal and, in fact, no significant change in growth was seen as a consequence of the treatment oils in any of the trials. If there was a reduction in total body fat as a result of the PUFAs in the diet, then other organs should also show an increase relative to body weight. Organ to organ weight ratios, therefore, are a better measure of specific changes in an organ under these circumstances. Indeed, liver:brain weight ratios in these studies reveal that there is no longer an observable effect of dose on liver weights in twelve of the thirteen studies (Table 7.5.9-4). The hypothesis that the change in relative liver weights is due to a reduced lipogenesis and body fat content would be consistent with the lack of histological or clinical chemical evidence for any liver toxicity. Literature reports also note that PUFAs are generally metabolized in the liver and the increased liver size in response to high doses of PUFAs, simply represents a natural hypertrophy of this organ to handle the increased metabolic load imposed upon it by the high doses of PUFA.

Table 7.5.9-5. The Effect of Dietary Supplementation With DHASCO and/or ARASCO at various doses (mg/kg/day) on the Liver/Brain wt Ratio in Male and Female Rats.

Males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				7.03	7.03	5.97	8.86	9.61				7.9	7.9
H	5.86	5.86	5.86	7.44	7.44	6.21	8.8	8.68	6.25	6.25	6.25	7.09	7.09
25	5.75												
50		5.36								7.18			
100								9.79	6.35				
200												6.89	
300										6.38			
500	5.97				7.46								
600								8.89	6.94				
1000		5.72		7.6						6.82		7.37	
1150							9.27						
1250	5.99				7.5								
1500			6.51							6.51			
1600						6.3							
2000									6.92				
2300								9.38					
2500		6.55		7.54									
3000									6.6		6.63		
3750			[redacted]										
4050							11.32						
4500						6.92					6.58		
4900												7.03	
8550							10.31						
9100													7.19
9250						6.71							

Female

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				4.17	4.17	4.56	5.3	5.32				4.4	4.4
0	4.47	4.47	4.47	4.28	4.28	4.96	4.93	5.47	4.42	4.42	4.42	4.15	4.15
25	4.32												
50		4.25								4.31			
100								5.77	4.37				
200												4.03	
300										4.05			
500	4.42				4.37								
600								5.63	4.2				
1000		4.77		4.19						4.52		4.34	
1150							4.99						
1250	4.4				4.34								
1500			4.63							4.45			
1600						4.53							
2000									4.29				
2300								6					
2500		4.53		4.66									
3000									4.28		4.52		
3750			5.08										
4050							5.96						
4500						4.69					4.46		
4900												4.44	
8550													
9100													4.9
9250						5.07							

Values in yellow represent the results of 13 separate toxicological studies; Highlighted values are statistically different from controls. Tox study labels indicate sponsor (MK, Martek; MJ, Mead Johnson; W, Wyeth/Ayerst; GB, Gist-brocades), duration (28 or 90 days), and test material (D, DHASCO; A, ARASCO; F, DHASCO+ARASCO; fo, fish oil). H, high fat control.

While carefully assessing liver weights, we also looked at changes in any other organ weights. No dose-related responses were found with any of the other organs except spleen. Like the liver, relative spleen weights were increased in only some of the studies, and the increased spleen weights were found only in the high dose groups. The spleen weight data are shown in Table 7.5.9-5 and Figure 7.5.9-2. Once again, the spleen weight changes were all well within the historical normal values, and there were no consistent dose-related responses. Furthermore, there were no significant changes when comparing spleen/brain weight ratios. With no associated histopathology or alterations in clinical chemistry, we again have no reason to disagree with the pathologists' reports that these findings are not adverse events. In many of the studies listed in Table 7.5.9-2, the authors also reported an increase in relative spleen weight in addition to the increases in relative liver weight. Again, the observation is made using equivalent doses of PUFA, regardless of the source of that PUFA, establishing that this is a PUFA-related phenomenon, and is independent of the carrier oil itself.

Table 7.5.9-5. The Effect of Dietary Supplementation With DHASCO and/or ARASCO at various doses (mg/kg/day) on the Relative Spleen Weights (% of body weight) of Male and Female Rats.

Males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				0.156	0.156	xx	0.175	xx				0.152	0.152
H	xx	xx	xx	0.155	0.155	xx		xx	0.187	0.187	0.187	0.159	0.159
25	xx												
50		xx								0.182			
100								xx	0.196				
200												0.15	
300										0.182			
500	xx				0.152								
600								xx	0.175				
1000		xx								0.195		0.152	
1150							0.165						
1250	xx				0.16								
1500			xx							0.201			
1600						xx							
2000									0.21				
2300								xx					
2500		xx											
3000											0.199		
3750			xx										
4050													
4500						xx					0.199		
4900												0.168	
8550													
9100													
9250						xx							

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				0.19	0.19	xx	0.192	xx				0.192	0.192
H	xx	xx	xx	0.199	0.199	xx	0.165	xx	0.206	0.206	0.206	0.182	0.182
25	xx												
50		xx								0.207			
100								xx	0.223				
200												0.18	
300										0.214			
500	xx				0.192								
600								xx	0.226				
1000		xx		0.233						0.217		0.181	
1150							0.187						
1250	xx				0.198								
1500			xx							0.222			
1600						xx							
2000									0.233				
2300								xx					
2500		xx		0.231									
3000									0.226		0.224		
3750			xx										
4050													
4500						xx							
4900													
8550													
9100													
9250						xx							

Values in yellow represent the results of 13 separate toxicological studies; Highlighted values are statistically different from controls. Tox study labels indicate sponsor (MK, Martek; MJ, Mead Johnson; W, Wyeth/Ayerst; GB, Gist-brocades), duration (28 or 90 days), and test material (D, DHASCO; A, ARASCO; F, DHASCO+ARASCO; fo, fish oil). H, high fat control; XX, values not measured.

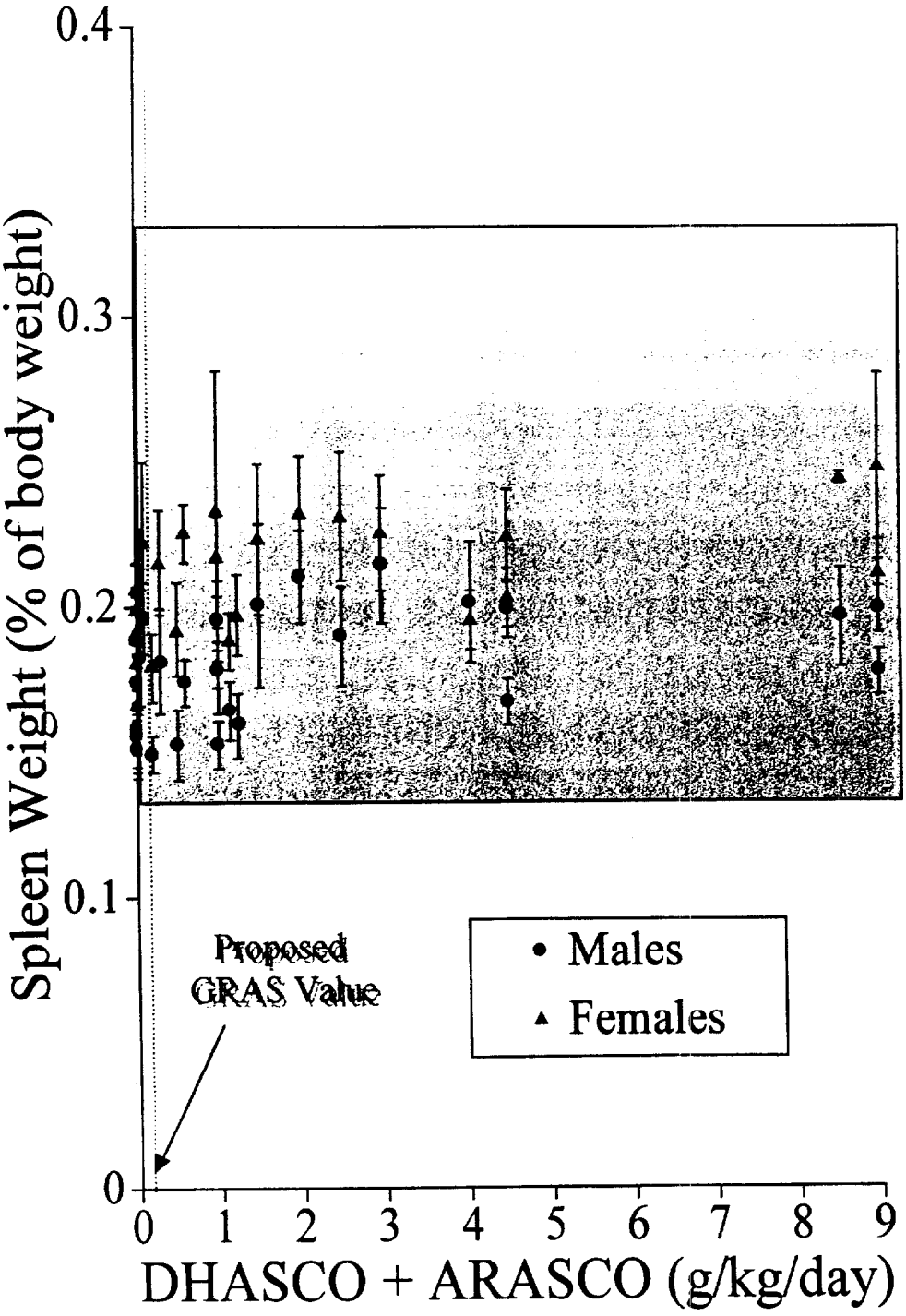

Figure 7.5.9-2. Changes in relative spleen weights in rats with chronic consumption of DHASCO and ARASCO. Shaded area represents the historical control range.

In conclusion, the administration of high doses of ARASCO, DHASCO or fish oil (more than 2 g/kg/day) to rats in a subchronic fashion can modestly increase liver and spleen weights relative to body weights. This effect largely takes place within a few

weeks of administration of the high levels of the PUFAs. Regardless of the source of PUFA (i.e., DHASCO, ARASCO, or fish oil) the magnitude of the response was similar when using similar levels of PUFA, and consistent with other reports in the literature with a wide variety of different fish oils. Thus, the relative liver and spleen weight changes appear to be a generalized LC-PUFA effect, and is not specific to either of the single cell oils.

The GRAS status of DHA and ARA is not the subject of this self affirmation, although this Panel cannot see how these PUFAs can be anything other than GRAS as they have been present in the diet (even in breast milk) as long as the evolutionary history of mankind. Rather, these genuine PUFA-related effects need to taken out of the consideration when establishing the safety of the sources of PUFA (*i.e.*, DHASCO and ARASCO) themselves. Since the DHA and ARA levels are defined by the intended, controlled use levels of the oils, the calculated safety margins should reflect the safety relative to the sources of the oils rather than the PUFAs themselves. For these reasons, we do not disagree with the various independent toxicologists conducting the safety studies in their interpretations that the NOAEL for the oils themselves represent the highest doses used in these studies. Such levels represent greater than a 50-fold excess over the intended use levels and are values for this macronutrient that would not be physically attainable in any sustained fashion.

7.6 PUFA POTENTIAL FOR OXIDATION. All polyunsaturated fatty acids are prone to oxygen radical attack. Such an interaction results in lipid peroxides that can cause severe injury to biological membranes. Nevertheless, the best nutritional source for a human infant is its mother's breast milk which effectively delivers DHA and ARA during early growth and development. Lipophilic antioxidants such as carotenoids or tocopherols exhibit a remarkable ability to prevent oxidative damage to biological membranes. In many cases, these lipophilic antioxidants act in concert with hydrophilic antioxidants such as ascorbic acid and glutathione. One antioxidant of major importance in the protection of oxidative damage within the cells is a large protein known as superoxide dismutase (SOD).

Infants, particularly preterm infants, are notoriously susceptible to metabolic disruptions caused by oxidative attack. Such pathologies as necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), and retinopathy of prematurity (ROP) are all thought to be aggravated by oxygen in preterm infants. Since human milk contains DHA and ARA, albeit at relatively low concentrations, one might expect *a priori* that human milk feedings might exacerbate the oxidation problem. On the contrary, however, it has long been known that these conditions generally occur at a lower frequency in infants who are receiving human milk (*i.e.*, with DHA and ARA) compared to those on artificial formulas. Recent biochemical evidence from several laboratories helps us to understand this phenomenon. It has been shown that when LC-PUFAs are incorporated into cellular membranes, there is a stimulation of the production of SOD (193, 194). Thus, the antioxidant status of an infant fed human milk may actually be better than that of a formula-fed infant because of the stimulation of the SOD by the DHA and ARA in the milk.

The above biochemical evidence explains why eliminating DHA and ARA from the diet of infants, particularly preterm infants, may put them at higher risk for oxidation dependent pathologies such as NEC, BPD and ROP. This has been recently confirmed in an animal model for NEC by Caplan *et al.* (38). Those authors demonstrated that the incidence of NEC was significantly lower in rats fed formulas containing DHA and ARA (using the DHASCO and ARASCO as sources) than in rats fed formulas containing no DHA or ARA. This has also been confirmed in human infants by Carlson and colleagues (42) using egg yolk as a source of DHA and ARA. In her controlled clinical study, infants who received standard preterm infant formula had an incidence of NEC of 18%, whereas the incidence was reduced to only 3% if the babies were given a formula that was supplemented with DHA and ARA. About 4,000 babies die each year in the United States as a result of NEC (35). Extrapolating Carlson's data to all U.S. babies, the inclusion of DHA and ARA into the formulas could substantially reduce the morbidity and mortality associated with this disease.

We conclude, therefore, that if DHASCO and ARASCO are provided to infants at the GRAS levels (*i.e.*, DHA and ARA up to 1.0% of total milk fat lipids), no oxidative injury would be expected. In fact, based on laboratory and clinical evidence, it is quite likely that the addition of DHA and ARA to formulas at these levels (similar to breast milk) may well reduce the incidence of oxidative damage in predisposed infants because of the stimulation of intracellular antioxidants such as SOD.

8. PRECLINICAL STUDIES.

There have been a number of reports involving the supplementation of the diets of various mammalian species including mice, rats, pigs, cats, dogs, monkeys and baboons with DHASCO and/or ARASCO. We are also aware of ongoing or unpublished studies with hamsters, cows, horses, and chickens. Some of these studies are tabulated and summarized in Appendix 1. Dosages used ranged from levels normally expected to be used as a dietary supplement, to doses wherein the total fat intake of the animal was comprised solely of DHASCO or ARASCO. None of these studies were designed as toxicology studies such as those described in Section 7, but rather, each of the investigators studied the effects of enriching tissue levels of DHA and/or ARA on a particular physiological response in a selected animal model. DHASCO and/or ARASCO are now commonly used as the dietary source of DHA and/or ARA by researchers because they are the most concentrated and purest sources available for use in such studies. These studies, in total, represent a very large experience base of dietary treatment of many different mammalian species with DHASCO and ARASCO, and there have been no suggestions from these reports of any toxicological or safety issues with these oils.

9. CLINICAL STUDIES

9.1 INFANT STUDIES. ARASCO and/or DHASCO oils have been the subject of at least 14 well-controlled clinical intervention trials involving approximately 1500 term or preterm infants, with over 700 of the infants receiving DHASCO and/or ARASCO oils (22, 41, 61, 91, 96, 112, 121, 236). These trials are summarized in Appendix 2. All of the trials analyzed blood lipids (either erythrocyte or plasma phospholipids) and growth as primary endpoints. In all cases, the supplementation of DHASCO and ARASCO resulted in an improvement of the circulating DHA and ARA status of the infant equivalent to that of a breast-fed infant and no study had any adverse effect on infant growth. One trial (61) was of particular interest in this respect because it involved the comparison of various doses of DHASCO and ARASCO to optimize the blood lipids of the infants. Other functional endpoints were also studied in a few cases. In contrast to preliminary reports of a reduced growth rate when using an EPA-containing (fish oil based) formula (45, 203), none of the studies reported reduced growth. In fact, two studies using DHASCO/ARASCO supplemented formulas report increased growth in the supplemented formula-fed infants (41, 77). As discussed in section 2.7, reduced growth rate in the fish oil trials may have been due to EPA, which is always present in fish oils, and the lack of added ARA to the formulas.

Significant improvements in visual and mental acuity have also been reported when infants were fed DHASCO/ARASCO supplemented formulas. Visual acuity improvements were reported to be equivalent to one line in an eye chart at one year of age (22). Mental acuity improvements in the same study were seven IQ points at 18 months of age, as determined by a Bayley MDI assessment[20]. In one preterm study (112) where visual acuity was assessed but no significant improvements were observed, the authors explain that the lack of a statistically significant response was likely due to the short duration of the supplementation (*ca.* only 4 weeks during the in-hospital stay). Another study did an extensive analysis of potential adverse events and the only statistically significant observations were that the DHASCO/ARASCO- supplemented formula-fed infants experienced less anemia and less nervousness or irritability (88,109, 236, 237). Although these would generally be categorized as beneficial aspects of the supplemented formula, the incidence levels were low and these were not primary endpoints of the study.

Finally, we were compelled by the data which indicates that infants fed standard formula had significantly altered blood biochemistry and a deficiency in visual and neurological functional assessments compared to breast-fed controls. *Post mortem* examination of the brains indicated brain DHA deficiencies as well (86, 164). Although the DHASCO/ARASCO-supplemented, formula-fed babies had significantly better functional outcomes than the standard formula-fed infants, they were never better than the breast-fed infants. Thus, re-supplying the DHA and ARA via the formula (using DHASCO and ARASCO) at the levels provided in breast milk is sufficient to overcome these deficiencies and the problems or biochemical abnormalities caused by feeding infants with a standard formula.

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[20] Presented by Dr. Birch at the Workshop of reference (217) and it is also in press (reference 21)

9.2 OTHER CLINICAL STUDIES. There have been at least 29 well-controlled, clinical intervention trials reported with adults, adolescents, or children wherein DHASCO and/or ARASCO have been used at various dose levels and for various periods of time (over 30 publications). These trials and relevant publications are summarized in Appendix 3 (note that some of these trials have led to multiple publications pertaining to various aspects of the trial). With respect to the safety of these oils, we have placed importance in the studies by Nelson (53, 136), since they were undertaken under the highly controlled environment of a metabolic ward by the United States Department of Agriculture. Furthermore, these studies used particularly high levels of supplementation – 3 g ARASCO per day (*ca.* 5% of daily fat) in the first trial (185), and 15 g DHASCO per day (*ca.* 25% of daily fat) in the second trial (187). A vast amount of data were gathered on the subjects (10 publications to date from these two studies). It was clear that the levels of DHA and ARA were significantly elevated in these subjects (*i.e.,* the material was bioavailable) and there were no reported adverse responses to the treatments. The other studies listed in Appendix 3 represent a cross section of healthy men, women and children of all ages, women who were pregnant or nursing, individuals with certain dietary restrictions (*i.e.,* vegetarians), and individuals with pre-existing metabolic disorders, including hyperlipidemia, retinitis pigmentosa, long chain hydroxyacylCoA dehydrogenase deficiency, ADHD, etc. The Nelson studies, in combination with the other studies using lower doses, provide additional evidence that the dietary supplementation of adults with DHASCO and/or ARASCO at the levels of 2-3 grams of oil per day would be generally recognized as safe by experts in the field.

10. COMMERCIAL USE OF DHASCO AND ARASCO

Since 1995, infant formulas containing DHASCO and ARASCO have been commercially produced and marketed around the world. At the present date, preterm formulas supplemented with DHASCO and ARASCO are available in at least 64 countries (Table 3.2). Full term formulas containing DHASCO and ARASCO are available in seven countries around the world. Although many of these countries did not require major safety evaluations and marketing approvals before commercialization, other countries, such as the UK, France and The Netherlands, did require an extensive evaluation before official approval to market the products was granted. We estimate that over 500,000 babies have now been fed formulas containing DHASCO and ARASCO, and there have been no reported adverse events associated with these products. This includes about 1,500 babies involved in 14 published and unpublished controlled clinical trials where even minor adverse events would have been carefully scrutinized (see Appendix 2). One company, Wyeth Ayerst, has been following their product launches with careful postmarket surveillance. Their specialty low birth weight formula (liquid and powder) is presently on the market in 56 countries around the world including Mexico, Australia, France, and the United Kingdom (Table 3-2). It has been on the market in some of these countries since 1997. This formula is provided under the careful supervision of doctors and the Company conservatively estimates that over 100,000 babies of most races, cultures and both sexes

have consumed the DHASCO/ARASCO supplemented formula and there has not been a single adverse event reported by the physicians.

Since 1997, DHASCO has also been commercially produced and marketed as a dietary supplement for adults (including women who are pregnant or lactating) in the United States under the tradename Neuromins®. Neuromins is a soft gelatin encapsulated form of the triglyceride DHASCO and is marketed by several different distributors in the US (Table 10-1). To date, the Company has sold over 35 million capsules for consumption. This product is primarily marketed in the United States, but it is now also available in Canada, Europe and Southeast Asia. We estimate that well over 500,000 individuals have consumed this product. To date there have been no adverse events reported to the Company with reference to the use of this product. This includes the nearly 20 well-controlled clinical trials where the appearance of any adverse events were carefully monitored (see Appendix 3).

The large numbers of individuals (infants, children and adults) who have consumed the commercial products containing DHASCO and/or ARASCO with no reported adverse effects provides additional support for the establishment of GRAS status for this product at the normal use levels.

Table 10-1. Commercial Products in the United States Containing DHASCO.

Manufacturer	*Trade Name*	*Product Form*
Martek	Neuromins	500 mg soft gel (100 mg DHA)
	Neuromins PL	500 mg soft gel (200 mg DHA)
	Neuromins for Kids	250 mg soft gel (100 mg DHA)
Solgar	Neuromins	500 mg soft gel (100 mg DHA)
Source Naturals	Neuromins	500 mg soft gel (100 mg DHA)
	Neuromins	500 mg soft gel (200 mg DHA)
	Focus Child	Chewable vitamin
Nature's Way	Neuromins	500 mg soft gel (100 mg DHA)
Solaray	Neuromins	500 mg soft gel (100 mg DHA)
BioDynamax	Neuromins	500 mg soft gel (100 mg DHA)
Whole Foods	Neuromins	500 mg soft gel (100 mg DHA)
Vitamin Shoppe	Neuromins	500 mg soft gel (100 mg DHA)
Your Life	Neuromins	500 mg soft gel (100 mg DHA)
Puritan's Pride	Neuromins	500 mg soft gel (100 mg DHA)
Omega Nutrition	Neuromins	500 mg soft gel (100 mg DHA)
Natrol	Neuromins	500 mg soft gel (100 mg DHA)
Safeway Select	Neuromins	500 mg soft gel (100 mg DHA)
Royal Body Care	EyeQ	500 mg soft gel (100 mg DHA)
KAL	Neuromins	Liquid Drop
Healthcom	UltraCare for Kids	Drink mix
Great Circles	Recovery	Bars and Drinks

11. CONCLUSIONS.

Based upon a critical evaluation and analysis of the information available on DHASCO and ARASCO, as summarized herein, the Panel has determined that these oils, derived from the referenced algal and fungal sources, meeting food grade specifications and produced according to current Good Manufacturing Practices (cGMP; 21 CFR 182.1) to be Generally Recognized as Safe (GRAS) by scientific procedures for use in supplementing the diets of infants and children at levels of 2.5% of dietary fat (1% of the diet by mass or 150 mg DHASCO (or ARASCO)/kg per day).

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